The Phosphate-Specific Transport System Gene *pstA1* Contributes to Rifampin Tolerance in *Mycobacterium tuberculosis*

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Keywords: *Mycobacterium tuberculosis*, PstA1, rifampin, antibiotic tolerance, phosphate-specific transport

Abstract

Tuberculosis (TB) caused an estimated 10.8 million new cases and 1.25 million deaths in 2023. Antibiotic tolerance, the ability of bacteria to survive exposure to bactericidal concentrations of antibiotics without genetic resistance mutations, contributes to the need for prolonged treatment. Adjunctive targeting of antibiotic tolerance mechanisms could promote accelerated clearance of *Mycobacterium tuberculosis* (*Mtb*), thereby improving medical adherence. Our previous forward genetic screen for rifampin tolerance genes identified *pstA1*, which is involved in phosphate-specific import. The ABC-type transporter permease PstA1 has previously been implicated in *Mtb* virulence, as mutants lacking this gene exhibit defective survival during phosphate limitation *in vitro*, in infected macrophages, and in immunocompetent mice. The minimum inhibitory concentration (MIC) of rifampin was not altered in a *pstA1* deletion mutant (Δ*pstA1*), suggesting this difference in susceptibility is not due to antibiotic resistance. Consistent with a role in rifampin tolerance, time-kill assays revealed a shift in the mean duration of killing (2-log reduction) from 1.6 days in wild-type to 1.0 day in Δ*pstA1*, and complementation partially restored this phenotype. We found that *pstA1* is specifically required for *Mtb* survival in the absence of exogenous inorganic phosphate and is important for adaptation to growth in culture without detergent, and within macrophages in an interferon-γ-dependent manner. Differential expression analysis revealed that Δ*pstA1* exhibited substantial transcriptional reprogramming with 58 differentially expressed genes (49 upregulated and 9 downregulated, adj. $P < 0.05$, $|\log_2 \text{ fold change}| > 1$), including altered expression of metabolic, DNA damage repair, and secretory pathways. PstA1 represents a novel drug target, and inhibitors could be used as adjunctive therapies to shorten curative treatment times, reducing opportunities for the emergence of drug resistance.

Importance

Tuberculosis remains one of the world's deadliest infectious diseases, causing over a million deaths annually. Current treatment requires months of antibiotic therapy, and poor adherence to these lengthy regimens contributes to the emergence of drug-resistant strains that are increasingly difficult to treat. A major barrier to shorter treatment is antibiotic tolerance, which allows bacteria to survive drug exposure without genetic resistance mutations. This study identifies the phosphate transport component PstA1 as a critical factor enabling *Mycobacterium tuberculosis* to tolerate the frontline antibiotic rifampin. Bacteria lacking *pstA1* are eliminated more rapidly by rifampin, demonstrating that this transporter actively promotes bacterial survival during treatment. These findings suggest that drugs targeting PstA1 could be combined with standard antibiotics to accelerate bacterial clearance, potentially shortening treatment duration and improving patient adherence. Such adjunctive therapies targeting tolerance mechanisms represent a promising strategy to combat tuberculosis and reduce the global burden of drug-resistant disease.

Introduction

Tuberculosis (TB) remains the leading cause of death from a single infectious agent, causing an estimated 10.8 million new cases and 1.25 million deaths in 2023 alone [1]. While effective regimens exist and three new drugs have been approved to treat TB since 2010, additional advances are needed to shorten treatment duration and improve treatment completion [2]. Current first-line TB treatment requires the use of multiple antibiotics for a minimum of six months to prevent relapse. A key reason for this lengthy treatment time is the presence of residual populations of bacteria that exhibit antibiotic tolerance– a phenotypic and reversible state in which bacteria become transiently insusceptible to antibiotics, requiring longer exposures to achieve bacterial eradication [3]. Bacteria can use a variety of adaptive mechanisms, collectively termed phenotypic tolerance, to adapt to and survive under stress conditions experienced within the host as well as antibiotic pressure [4]. The molecular mechanisms underlying antibiotic tolerance in *Mtb* are complex, involving transcriptional stress responses, metabolic adaptations, activation of drug efflux pumps, and changes in cell wall permeability [5]. Direct disruption

of *Mtb* antibiotic tolerance mechanisms is expected to result in more rapid bacillary killing, leading to shorter treatment times that may improve medical adherence and decrease opportunities for the development of antibiotic resistance [6].

We previously used transposon mutant sequencing (Tn-seq) to identify *Mtb* genes required for survival during exposure to bactericidal concentrations of the first-line drug rifampin. Among the top hits from this unbiased genome-wide approach were the phosphate-specific transport (Pst) genes *rv0820/phoT*, *rv0929/pstC2*, and *rv0930/pstA1* [7]. Notably, their homologs were also identified in our similar forward genetic screen in which *M. avium* was exposed to rifabutin, providing additional confidence in their significance and suggesting that Pst genes contribute to rifamycin tolerance across mycobacterial species [6]. Pst systems are comprised of four essential components: PstA and PstC are permeases that form a transmembrane channel complex; PstS is an extracellular protein that senses and captures phosphate from the environment; and PstB is an intracellular ATPase that provides energy for active transport of phosphate against its concentration gradient [8-10]. PstA1, which is co-expressed as part of the putative *Mtb* operon *pstS3-pstC2-pstA1* [11], is an important virulence factor required for *Mtb* survival during acidic stress [12] and phosphate depletion [13, 14], as well as for *Mtb* survival within macrophages [15] and for resistance to IFN-γ dependent immunity in mice [14]. PstA1 also plays important regulatory roles, as several substrates of the ESX-5 secretion system, as well as membrane vesicles, are hypersecreted in Δ*pstA1* mutants [16, 17]. Each of these phenotypes is mediated at least in part through the constitutive activation of RegX3, a response regulator of the PhoPR two-component system, in the absence of PstA1 [18, 19]. This regulatory connection links phosphate sensing to broader stress responses and virulence factor expression. Previous studies have shown that a *pstA1* deletion mutant was more susceptible to several drug combinations *in vitro* and to rifampin in a mouse model [20], providing initial evidence for a role in antibiotic tolerance. However, the molecular mechanisms underlying this phenotype have not been fully elucidated.

In this study, we have comprehensively characterized the role of *pstA1* in rifampin tolerance using phenotypic assays, transcriptomics, and metabolomics to explore the

115 underlying molecular mechanisms of its increased susceptibility. We have also studied
116 the role of PstA1 in *Mtb* growth and survival in phosphate-rich and phosphate-depleted
117 models of nutrient starvation and within macrophages, providing insights into how
118 phosphate transport contributes to bacterial adaptation under diverse stress conditions.
119 Our findings reveal new mechanistic insights into antibiotic tolerance and highlight the
120 potential for targeting phosphate transport pathways in TB treatment.

121 **Results**

122 **Phosphate transport gene deletions confer rifampin hypersusceptibility**

123 Our Tn-seq screen to identify *Mtb* genes required for tolerance to the sterilizing drug
124 rifampin identified three Pst genes, *rv0820/phoT*, *rv0929/pstC2*, and *rv0930/pstA1*,
125 among the top hits [7]. Their homologs were also found in a similar Tn-seq study of *M.*
126 *avium* exposed to rifabutin [6]. Notably, *pstC2* and *pstA1* are downstream components of
127 an *Mtb* operon including *pstS3* [22, 37]. To confirm that these three genes are co-
128 transcribed in the H37Rv strain under our experimental conditions, we performed RT-
129 PCR analysis using primers spanning the intergenic and intragenic regions of this operon.
130 Our results confirmed that *pstS3*, *pstC2*, and *pstA1* are co-expressed as a single
131 polycistronic mRNA transcript (Fig 1), validating the operonic structure for subsequent
132 studies.

133 Next, we generated individual targeted deletion strains for each of these three genes
134 using the ORBIT cloning system [21]. Cloning was validated via PCR of the 5' and 3' ends
135 of the insertion site, as well as with whole genome sequencing for Δ*pstA1* (SeqCenter,
136 Pittsburgh, PA) (Supplementary Figure S1). To validate our Tn-seq findings, we assessed
137 the activity of rifampin against each deletion strain using standard time-kill assays
138 following addition of 2.0 µg/ml rifampin. All three deletion strains showed increased
139 susceptibility to rifampin treatment compared to wildtype controls harboring empty
140 vectors. At day 6, Δ*phoT* showed a non-significant 0.9 $\log_{10}$ reduction in CFU compared
141 to control (adjusted P = 0.358; Fig. 2A). Δ*pstC2* demonstrated a more pronounced
142 phenotype with an additional 1.3 $\log_{10}$ reduction (adjusted P = 0.000189, Fig. 2B). Δ*pstA1*

143 exhibited the strongest susceptibility to rifampin, showing an additional 1.9 $\log_{10}$ reduction
144 in viable bacteria (adjusted P = 0.00742) relative to the isogenic wild-type control (Fig.
145 2C). The minimum duration of killing (MDK), defined as the time required to achieve a 2-
146 $\log_{10}$ reduction from the starting bacterial density, decreased by 0.4 days for Δ*phoT*, 1.1
147 days for Δ*pstC2*, and 0.6 days for Δ*pstA1* relative to controls. Consistent with a role for
148 each of these genes in *Mtb* tolerance, rather than resistance, to rifampin, each deletion
149 mutant showed minimal alterations in rifampin MIC values. The MIC values of rifampin
150 against Δ*phoT* and Δ*pstC2* were 0.03 µg/ml and that of Δ*pstA1* was 0.016 µg/ml (wild
151 type, 0.03-0.06 µg/ml).

152 **Complementation of *pstA1* partially restores wild-type rifampin tolerance**

153 Given the robust phenotype observed with Δ*pstA1*, and the possibility that deletion of
154 *pstC2* could have polar effects on *pstA1*, our subsequent studies focused on Δ*pstA1*. To
155 validate the *pstA1* deletion phenotype, we constructed a genetic complementation strain
156 (Δ*pstA1::pstA1*). Since *pstS3*, *pstC2*, and *pstA1* are co-transcribed as an operon, we
157 designed our complementation construct to include appropriate regulatory elements. The
158 *pstA1* coding sequence fused to the putative operonic promoter region (422 bp upstream
159 of *pstS3* plus the first 75 bp of *pstS3*) was cloned into the episomal mycobacterial vector
160 pMH94 and introduced into the Δ*pstA1* strain. Complementation was confirmed by PCR
161 of insert junctions and whole genome sequencing (Supplementary Figure S1). Time-kill
162 assays revealed that the minimum duration of killing (MDK) of rifampin for the
163 complement strain was 1.3 days, as compared to 1.6 days for the wild type and 1.0 day
164 for ΔpstA1 (Fig. 2D). MDK values differed significantly between all three strains (WT vs
165 ΔpstA1: p = 0.00004; WT vs pstA1 comp: p = 0.004; ΔpstA1 vs pstA1 comp: p = 0.0011,
166 one-way ANOVA with Tukey's multiple comparisons test). Thus, genetic complementation
167 of Δ*pstA1* partially restored wild-type rifampin tolerance.

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170 **Δ*pstA1* exhibits phosphate-specific survival defects while maintaining rifampin**
171 **susceptibility under starvation conditions**

172 To characterize the broader physiological role of *pstA1*, we examined bacterial survival
173 under various stress conditions relevant to mycobacterial pathogenesis and persistence.
174 As multiple forms of nutrient starvation are known to induce a tolerance phenotype [13,
175 22, 23], we tested the ability of Δ*pstA1* to survive in both the PBS nutrient starvation model
176 used in our prior screen [7] and during inorganic phosphate limitation [13].

177 During nutrient starvation in phosphate-buffered saline containing the detergent tween-
178 80, both wild-type and Δ*pstA1* strains showed equivalent survival over a 14-day
179 adaptation period, with no significant differences observed between strains (Fig. 3A).
180 *ΔpstA1* retained enhanced rifampin susceptibility under nutrient-starved conditions.
181 When rifampin (2.0 µg/ml) was added after the 14-day starvation period, the deletion
182 mutant showed significantly enhanced killing compared to wild-type, with additional
183 reductions in viability becoming apparent by day 2 (1.4 $\log_{10}$ reduction; adjusted P =
184 0.000184) and persisting through day 6 (1.6 $\log_{10}$ reduction; adjusted P = 0.000184) (Fig.
185 3B).

186 Since phosphate depletion is known to induce the stringent response, a key antibiotic
187 tolerance pathway in *Mtb* [24, 25], and the *pstS3-pstC2-pstA1* operon and *phoT* are
188 upregulated under low phosphate conditions [13], we tested the ability of Δ*pstA1* to
189 survive in the absence of exogenous inorganic phosphate. Phosphate-specific depletion
190 revealed a clear survival disadvantage for Δ*pstA1*. While both wild-type and Δ*pstA1*
191 strains exhibited similar growth and survival characteristics in standard phosphate-rich
192 7H9 broth, Δ*pstA1* showed significantly reduced viability under phosphate-depleted
193 conditions. In modified 7H9 broth lacking inorganic phosphate, the deletion strain
194 demonstrated an additional survival defect with a 0.7 $\log_{10}$/ml decrease by day 7 (adjusted
195 P = 0.0115) and a 0.6 $\log_{10}$/ml decrease by day 14 (adjusted P = 0.00181) relative to wild
196 type (Fig. 3C). These results confirm the role of *pstA1* in phosphate acquisition under
197 limiting conditions and demonstrate its requirement for growth in low-phosphate
198 environments.

199 **Δ*pstA1* exhibits survival defects in IFN-γ-activated macrophages**

200 To assess the relevance of our findings to mycobacterial pathogenesis, we next examined
201 the role of *pstA1* in macrophage infection and intracellular survival as a model of the
202 natural host environment. Macrophages represent the primary host cell niche for *Mtb* and
203 create a hostile intracellular environment characterized by nutrient limitation, acidic pH,
204 and antimicrobial factor exposure [15]. We infected PMA-differentiated THP-1
205 macrophages with wild-type and Δ*pstA1* strains at a multiplicity of infection (MOI) of 1:1
206 and monitored intracellular bacterial survival over 4 days. In resting macrophages, there
207 was no significant difference in bacterial burden between wild-type and Δ*pstA1* at any
208 time point measured (days 0, 2, and 4 post-infection; Fig. 4A), suggesting that *pstA1* is
209 not required for initial invasion or survival in resting macrophages. However, when
210 macrophages were pre-activated with IFN-γ, a key cytokine that enhances macrophage
211 antimicrobial functions, Δ*pstA1* showed a significant survival defect that became
212 pronounced by day 4 post-infection. While wild-type bacteria maintained relatively high
213 intracellular numbers (4.2×10^6 CFU/ml), the Δ*pstA1* strain showed reduced survival
214 (2.43×10^6 CFU/ml), representing a statistically significant difference (adjusted P = 0.0098;
215 Fig. 4B). This result confirms previous reports that *pstA1* is specifically required for
216 resistance to IFN-γ-mediated host immunity and suggests a role in counteracting
217 activated macrophage antimicrobial mechanisms [15].

218 **The contribution of PstA1 to *Mtb* rifampin tolerance is not mediated through the**
219 **accumulation of inorganic polyphosphate**

220 Having established that Δ*pstA1* exhibits enhanced rifampin susceptibility, we next sought
221 to investigate the underlying mechanisms responsible for this phenotype. Induction of the
222 *Mtb* stringent response and accumulation of the multifunctional stress signaling molecule,
223 inorganic polyphosphate (polyP), has been linked to antibiotic tolerance in *Mtb* [26-30].
224 Since RegX3, a two-component response regulator that controls the *Mtb* phosphate
225 starvation and stringent responses [13, 31], is constitutively activated upon *pstA1* deletion
226 [14], we measured intracellular polyP levels as a proxy for stringent response activation.
227 No significant differences in polyP accumulation were observed between wild-type and

228 Δ*pstA1* strains across different growth phases (Fig. 5A). Wild-type polyP content ranged
229 from 3.072 to 5.536 ng/µg total protein, while Δ*pstA1* levels ranged from 3.673 to 5.915
230 ng/µg total protein.

231 **Δ*pstA1* exhibits increased rifampin uptake**

232 We hypothesized that *pstA1* deficiency might be associated with increased intrabacterial
233 accumulation of rifampin. Using a previously validated device that enables timed start-
234 stop measurements of antibiotics remaining in spent media [32], we quantified rifampin
235 content in the wild-type and mutant strains The kinetics of rifampin uptake revealed that
236 Δ*pstA1* displayed significantly faster and greater rifampin accumulation compared to wild-
237 type at 6 hours post-exposure, suggesting increased uptake and/or decreased efflux of
238 rifampin (Fig. 5B-C).

239 **Cell morphology analysis reveals minimal structural differences**

240 To determine whether the observed increase in rifampin uptake was attributable to gross
241 alterations in cell wall structure of the mutant, we examined bacterial morphology using
242 electron microscopy. Under standard growth conditions including Tween-80 to prevent
243 bacterial clumping, we observed no significant difference in cell length between the two
244 strains (average bacterial length= 2.34 µm for wild type and 2.26 µm for Δ*pstA1;* Fig. 5D).
245 Likewise, the strains showed similar average cell wall thicknesses of 21.18 nm and 19.61
246 nm, respectively (Fig. 5E). Substantial variations in cell wall thickness were observed for
247 both wild-type and Δ*pstA1* which appear similar to previously reported lipid inclusions
248 [33]. There were no significant differences in minimum, maximum, or average cell wall
249 thickness between the two strains.

250 Since use of detergent can alter cell wall permeability [34], the experiment was repeated
251 using cultures grown without Tween-80 (Fig. 5F-G). Cell length decreased for both strains
252 in detergent-free broth, from an average of 2.34 µm to 1.64 µm for wild-type and from
253 2.26 µm to 1.95 µm for Δ*pstA1* (difference= 0.31 µm; P = 0.0329; Fig. 5F). Cell wall
254 thickness did not vary notably between strains with averages of 15.37 nm for wild type
255 and 16.06 nm for Δ*pstA1*. The large variations in cell wall thickness seen in cultures grown

with Tween-80 were not present under detergent-free conditions (Fig. 5H). These results demonstrate that Δ*pstA1* exhibits increased rifampin accumulation without major changes in cellular morphology.

Transcriptomic analysis reveals extensive metabolic and regulatory reprogramming in Δ*pstA1*

To further explore the molecular mechanisms underlying the increased rifampin susceptibility of Δ*pstA1* and identify potential cellular pathways contributing to enhanced drug uptake, we performed transcriptomic analysis using RNA sequencing. Wild-type and Δ*pstA1* strains were exposed to a sub-inhibitory concentration of rifampin (7.5 ng/ml, representing 1/4 MIC) for 30 or 60 minutes. This concentration and timing were selected to capture early transcriptional responses while minimizing the global transcriptional inhibition that occurs at bactericidal concentrations of rifampin. During axenic growth in nutrient-rich broth lacking rifampin, Δ*pstA1* exhibited substantial transcriptional reprogramming compared to wild type, with 58 differentially expressed genes (DEGs) identified: 49 upregulated and 9 downregulated (adjusted $P < 0.05$, $|\log_2 \text{fold change}| > 1$; Fig. 6A). Several genes known to be part of the phosphate regulon showed significant upregulation, including *pstS3* ($\log_2$ fold change = 3.22), *pstC2* ($\log_2$ fold change = 2.50), and the two-component regulatory system genes *senX3* ($\log_2$ fold change = 1.59) and *regX3* ($\log_2$ fold change = 1.77), indicating constitutive activation of phosphate starvation signaling.

KEGG pathway enrichment analysis revealed that genes involved in biosynthesis and metabolic pathways were prominently represented among the DEGs (Fig. 6B). The differentially expressed genes included fourteen PE/PPE family genes (9 upregulated, 5 downregulated), with upregulation of members of the ESX-5 locus and downregulation of *pe31* ($\log_2$ fold change = -6.02) and *ppe60* ($\log_2$ fold change = -3.37). Genes encoding four ESAT-6-like proteins were upregulated, namely *esxI* ($\log_2$ fold change = 3.65), *esxJ* ($\log_2$ fold change = 2.59), esxV ($\log_2$ fold change = 3.14), and *esxW* ($\log_2$ fold change = 2.35). The redox-responsive transcriptional regulator *whiB3* ($\log_2$ fold change = 2.60), a key regulator of thiol metabolism and oxidative stress responses, was also significantly

upregulated [12]. Additionally, eleven genes involved in DNA metabolism were upregulated in the mutant at baseline, including resolvases, transposases, and DNA helicases. Rifampin treatment led to a progressive reduction in the number of DEGs between strains. After 30 minutes of rifampin exposure, 41 genes remained differentially expressed between strains. After 60 minutes, 41 genes were differentially expressed, with 36 genes showing consistent differential expression across all three conditions (baseline, 30 min, and 60 min). A comprehensive Venn diagram analysis of DEG overlap across all treatment conditions is shown in Supplementary Figure S2. The DNA metabolism genes were no longer significantly differentially expressed following rifampin treatment, while the core set of 36 genes included the phosphate regulon genes, all four ESAT-6-like protein genes, twelve PE/PPE family genes, and *whiB3*.

RT-qPCR analysis of 11 selected genes confirmed the expression patterns observed in the RNA-seq dataset, with correlation coefficients exceeding 0.7 for all comparisons (Fig. 6D; Supplementary Figure S3). Validated genes included members of the phosphate starvation response (*pstC2*, $\log_2$ fold change = 2.50; *regX3*, $\log_2$ fold change = 1.77; *senX3*, $\log_2$ fold change = 1.59), metabolic enzymes (*udgA*, $\log_2$ fold change = 3.08; *mgtA*, $\log_2$ fold change = 3.04), the stress response regulator *whiB3* ($\log_2$ fold change = 2.60), and PE/PPE family protein genes (*ppe60*, $\log_2$ fold change = -3.37; *ppe65*, $\log_2$ fold change = 1.89). A complete list of all DEGs across all experimental conditions is provided in Supplementary Table S2.

Metabolomic analysis reveals altered antioxidant metabolites in Δ*pstA1*

To understand the biochemical consequences of increased rifampin accumulation in the deletion mutant, we performed targeted metabolomics analysis of rifampin-treated bacteria and analysis was focused on antioxidant pathways. We analyzed the intracellular abundance of 16 metabolites involved in antioxidant biosynthesis and redox homeostasis in the Δ*pstA1* mutant, wild-type, and complement strains at 6 hours post-rifampin exposure. The heatmap analysis of all measured antioxidant metabolites revealed distinct metabolic profiles between strains (Fig. 7A). While most metabolites, including L-glutamate, glycine, NADPH, glutathionylspermidine, and various glutathione derivatives,

314 showed trends toward increased abundance in Δ*pstA1*, these changes did not reach
315 statistical significance. The complement strain generally displayed metabolite levels
316 which were intermediate between wild-type and the deletion mutant, consistent with
317 partial restoration of the metabolic phenotype.

318 Following rifampin treatment, two antioxidant pathway metabolites showed trends toward
319 altered abundance in Δ*pstA1* compared to wild-type: ergothioneine and 5-oxoproline ($p <$
320 0.1, ANOVA; Fig. 7B). Ergothioneine, a sulfur-containing antioxidant, showed a trend
321 toward elevation in Δ*pstA1* compared to both wild-type and complement strains. This
322 trend is notable given that *whiB3*, which was upregulated 2.6-fold in the transcriptomic
323 analysis and confirmed by RT-qPCR, is a metabolic regulator involved in the production
324 of ergothioneine and mycothiol, which play an important role in detoxifying the oxidative
325 stress [35]. Similarly, 5-oxoproline, a glutamate derived metabolite, showed a trend
326 toward increased abundance in the deletion mutant [36]. The observed trends suggest
327 that Δ*pstA1* may exhibit hypersensitivity to rifampin largely because of alterations in
328 antioxidant metabolism following increased rifampin exposure.

329 **Discussion**

330 Components of the Pst system have been implicated in virulence, stress tolerance, and
331 antibiotic susceptibility in several bacterial pathogens, suggesting that phosphate-specific
332 transport pathways may represent promising targets for therapeutic intervention [14, 37].
333 This study establishes PstA1 as a critical determinant of rifampin tolerance in *Mtb* through
334 mechanisms distinct from drug resistance. Deletion of *pstA1* resulted in enhanced
335 rifampin killing without substantial change in MIC, a phenotype that remained consistent
336 across nutrient starvation conditions and correlated with reduced survival in IFN-γ-
337 activated macrophages. Integrative multi-omics analysis revealed that *pstA1* loss triggers
338 constitutive phosphate starvation signaling, leading to PE/PPE porin upregulation. These
339 findings raise the possibility that PE/PPE porin upregulation enhances membrane
340 permeability, thereby promoting rifampin uptake. Metabolic profiling revealed
341 nonsignificant upregulation of antioxidant pathways including ergothioneine and

342 glutathione biosynthesis, suggesting elevated oxidative stress resulting from increased
343 intracellular rifampin accumulation due to hyperpermeability [35].

344 In contrast to prior studies showing that *pstA1* deficiency reduces the MIC of rifampin by
345 8-fold, we found a more modest 2-fold decrease in the rifampin MIC against Δ*pstA1*. This
346 discrepancy may be due to differences in the parent strain used (Erdman vs. H37Rv) [20].
347 Notwithstanding these findings, we observed significantly increased rifampin activity
348 against Δ*pstA1* in time-kill assays, the standard approach for assessing antibiotic
349 tolerance [5].

350 Δ*pstA1* retained its rifampin hypersusceptibility under nutrient starvation and showed
351 reduced survival under phosphate depletion, consistent with previous reports [14, 15] and
352 validating its core metabolic function in phosphate acquisition. This metabolic vulnerability
353 extended to host-pathogen interactions. While a previous Tn-seq study found that *pstS3*,
354 *pstC2*, *pstA1*, and *phoT* were each important for macrophage survival under all activation
355 conditions tested [15], Δ*pstA1* showed a more nuanced phenotype in the current study,
356 with no survival defect in resting macrophages but significant reduction in intracellular
357 survival in IFN-γ-activated macrophages by day 4. This finding aligns well with that from
358 a separate study reporting that Δ*pstA1* retained full virulence in IFN-$\gamma^{-}/^{-}$ mice but showed
359 reduced chronic-phase survival in wild-type mice, establishing that PstA1 is specifically
360 required for surviving IFN-γ-mediated immunity [14].

361 To understand the mechanistic basis for enhanced rifampin susceptibility, we examined
362 drug uptake dynamics and cellular structure. We observed accelerated rifampin uptake
363 with significantly elevated drug accumulation by 6 hours, implicating increased drug
364 permeability and/or reduced efflux. Electron microscopy revealed no major structural
365 changes in cellular morphology. Our transcriptomic analysis provided insight into the
366 molecular basis for this altered permeability state. In the absence of rifampin exposure,
367 Δ*pstA1* showed extensive cellular reprogramming with 58 genes differentially expressed.
368 Most striking was the constitutive activation of the phosphate starvation regulon, including
369 *pstS3*, *pstC2*, *senX3*, and *regX3,* indicating that cells lacking functional PstA1 exist in a
370 state of perceived phosphate limitation regardless of environmental phosphate

371 availability. The marked upregulation of *pstS3* and *pstC2* likely represents compensatory
372 transcriptional upregulation consistent with constitutively activated RegX3, as multiple
373 studies have demonstrated RegX3-dependent regulation of the *pstS3-pstC2-pstA1*
374 operon [13, 14, 18]. Without the functional PstA1 permease, however, this transcriptional
375 response cannot restore phosphate transport, resulting in persistent phosphate starvation
376 signaling.

377 This baseline stress state likely compromises bacterial ability to respond effectively to
378 additional challenges like antibiotic exposure. There is substantial concordance between
379 our transcriptomic data and those of another study evaluating a *pstA1* deletion mutant in
380 the Erdman background, as 22 common DEGs were also identified in both studies,
381 including *senX3*, *regX3*, the *pstS3-pstC2-pstA1* operon, *whiB3*, and 9 PE/PPE genes,
382 validating the core transcriptional signature of *pstA1* deficiency across strain backgrounds
383 [14].

384 Among the 36 genes differentially expressed in *ΔpstA1* regardless of rifampin treatment,
385 12 encoded PE/PPE family genes and 4 encoded ESAT-6-like proteins. Four of these
386 PE/PPE genes (PE18, PPE19, PPE25, PPE27) are part of the *esx-5* locus, consistent
387 with established regulation of ESX-5 secretion by the Pst/SenX3-RegX3 axis and the
388 finding that *pstA1* deletion causes ESX-5 substrate hypersecretion. This upregulation
389 provides a direct mechanistic link to the increased rifampin uptake we observed [16].
390 Recent work demonstrated that Δ*pstA1* mutants overcome acid growth arrest by
391 upregulating PE19-PPE51 porin expression to enhance glycerol uptake [12], while
392 another study established that PE/PPE proteins function as substrate-specific outer
393 membrane channels [38]. The constitutive overexpression of multiple PE/PPE proteins in
394 Δ*pstA1* facilitates not only glycerol uptake but also rifampin entry through increased
395 membrane permeability, potentially explaining the accelerated drug accumulation and
396 enhanced killing [12]. Transcriptomic analysis revealed no downregulation of known
397 rifampin efflux pump genes, further supporting the hypothesis that enhanced drug
398 accumulation results from increased membrane permeability rather than impaired efflux.
399 The absence of gross structural defects on electron microscopy supports the

interpretation that increased permeability results from specific changes in channel composition rather than general membrane disruption.

Interestingly, DNA-interacting genes were only upregulated in Δ*pstA1* in the absence of rifampin, suggesting that drug's inhibitory effects on RNA polymerase may suppress some baseline transcriptional differences. We identified only a few genes differentially regulated exclusively upon rifampin exposure, likely because of the relatively low drug concentration and brief timepoints, which were selected to minimize global transcriptional inhibition.

Our integrated metabolomic and transcriptomic analysis revealed activation of antioxidant pathways in rifampin-treated Δ*pstA1*, consistent with elevated oxidative stress from increased intracellular drug accumulation. We observed an upward trend of metabolites involved in antioxidant defense, including 5-oxoproline, glutamate, glutathionylspermidine, and notably ergothioneine. The ergothioneine response is particularly informative: this mycobacterial antioxidant provides protection against rifampin-induced damage, as a study demonstrated that Δ*egtD* mutants lacking ergothioneine show enhanced susceptibility to rifampin and other first-line drugs [39]. The apparent paradox in Δ*pstA1*, where ergothioneine is elevated yet susceptibility increases, could reflect compensatory antioxidant upregulation that is insufficient to overcome the primary defect of PE/PPE-mediated drug hyperpermeability.

The concurrent 2.6-fold upregulation of *whiB3*, a redox-responsive regulator of thiol metabolism, further supports activation of oxidative stress defenses. While one study showed that WhiB3 negatively regulates ergothioneine biosynthesis under standard conditions, the simultaneous increase in both *whiB3* expression and ergothioneine levels in rifampin-treated Δ*pstA1* suggests that rifampin-induced oxidative stress may trigger compensatory antioxidant production [35]. The elevation of 5-oxoproline, a glutamate-derived metabolite, indicates broad activation of thiol-based defenses beyond ergothioneine alone [40]. Critically, despite these activated protective pathways, Δ*pstA1* exhibits marked hypersusceptibility to rifampin, suggesting that the primary driver of enhanced killing is increased drug accumulation rather than deficient antioxidant capacity.

429 This is further supported by recent findings showing that WhiB3 expression is induced by
430 both acidic pH and phosphate limitation through RegX3-dependent mechanisms, and that
431 elevated WhiB3 levels in Δ*pstA1* correlate with reduced acid-induced ROS and preserved
432 GAPDH activity [12].

433 While increased rifampin uptake provides mechanistic connection to enhanced killing, the
434 molecular basis for altered drug accumulation requires further investigation. The link
435 between PE/PPE upregulation, ESX-5 hypersecretion, and rifampin permeability needs
436 direct demonstration. The transcriptomic analysis captures a snapshot of cellular
437 responses and may miss dynamic changes occurring at other timepoints. The targeted
438 metabolomics, while revealing specific antioxidant pathway alterations, can be extended
439 to look at relevant perturbations in central metabolism or lipid composition that could
440 contribute to the phenotype.

441 The identification of *pstA1* as a tolerance determinant opens potential therapeutic
442 avenues, and targeting this pathway requires careful consideration. Our findings show
443 that loss of PstA1 function creates multiple vulnerabilities, including enhanced drug
444 uptake, metabolic dysregulation, and impaired stress responses, suggesting that
445 constitutive activation of phosphate-sensing pathways during antibiotic treatment
446 becomes a liability rather than an adaptive advantage. The specific mechanisms identified
447 are intriguing targets: the WhiB3-ergothioneine stress response axis and PE/PPE-
448 mediated membrane changes represent downstream effectors that could be modulated
449 without disrupting fundamental phosphate homeostasis. Understanding how phosphate
450 transport influences antibiotic tolerance could inform adjunctive strategies to eliminate
451 persister populations, potentially shortening the lengthy treatment regimens currently
452 required for TB cure.

453

454

455

Materials and Methods

Bacterial Strains and Plasmids

Mycobacterium tuberculosis H37Rv served as the wild-type strain for all experiments. Gene deletion strains were constructed using ORBIT (Oligonucleotide-Recombineering followed by Bxb1 Integrase Targeting) cloning as previously described [94]. Wild-type H37Rv was first transformed with pKM444, a non-integrating plasmid encoding RecT recombinase and Bxb1 integrase (gift from Kenan Murphy, University of Massachusetts). In a second electroporation, cells received pKM464 (also from Kenan Murphy) along with a gene-specific oligonucleotide designed to delete pstA1. This oligonucleotide contained the first and last 11 codons of pstA1 flanked by 37-bp homology arms, with the Bxb1 attP integration site positioned centrally. Transformants were selected on antibiotic-selective plates, and deletion junctions were verified by PCR amplification followed by Sanger sequencing.

For complementation, the pstA1 coding sequence plus its upstream regulatory region (422 bp upstream of pstS3 plus the first 75 bp of pstS3) was PCR-amplified from wild-type H37Rv genomic DNA. This fragment was cloned into pMH94, an episomal mycobacterial vector modified with apramycin resistance (gift from Dr. Gyanu Lamichhane, Johns Hopkins University), using In-Fusion Snap Assembly (Takara Bio). The vector was first linearized by restriction digestion with EcoRI and XbaI, gel-purified using QIAquick Gel Extraction Kit (Qiagen), then assembled with the PCR product. Cloned constructs were transformed into Stellar Competent Cells (Takara Bio), selected on LB plates with appropriate antibiotics, and verified by PCR before electroporation into the ΔpstA1 strain.

Culture Conditions and Media

M. tuberculosis strains were routinely cultured in Middlebrook 7H9 broth supplemented with 10% OADC enrichment, 0.2% glycerol, and 0.05% Tween-80, or on Middlebrook 7H11 agar plates incubated at 37°C. Phosphate-depleted medium was prepared as described previously [24] by omitting inorganic phosphate salts from the 7H9 formulation.

484 THP-1 monocytic cells were maintained in RPMI-1640 medium (Gibco) containing 2 mM
485 L-glutamine and 10% heat-inactivated fetal bovine serum at 37°C with 5% CO_2.

486 **Operon Structure Validation**

487 To confirm co-expression of *pstS3*, *pstC2*, and *pstA1* as a single operon, RNA was
488 extracted from logarithmically growing *Mtb* H37Rv cultures using TRIzol reagent.
489 Following DNase-I treatment to eliminate genomic DNA contamination, RNA was reverse
490 transcribed using SuperScript VILO cDNA Synthesis Kit (Invitrogen). PCR primers were
491 designed to amplify both intragenic regions (within individual genes) and intergenic
492 regions (spanning gene boundaries).

493 **Whole Genome Sequencing Validation**

494 High-molecular-weight genomic DNA was extracted from 10 mL log-phase cultures using
495 an extended protocol optimized for mycobacteria. Cultures were lysed by sequential
496 treatments with lysozyme (20 hours at 37°C), SDS/proteinase K (50°C), and CTAB,
497 followed by chloroform/isoamyl alcohol extraction and isopropanol precipitation. To verify
498 deletion and complementation, genomic DNA from wild type, Δ*pstA1*, and complement
499 strains were submitted to SeqCenter (Pittsburgh, PA) for whole genome sequencing.
500 Libraries were prepared using the Illumina DNA Prep kit with tagmentation-based
501 fragmentation and sequenced on an Illumina NovaSeq X Plus platform (2×151 bp paired-
502 end reads). Raw sequencing reads were aligned to the *M. tuberculosis* H37Rv reference
503 genome, and coverage analysis was performed using SAMtools v1.18. Deletion
504 boundaries were confirmed by examining read coverage across the *pstA1* locus.

505 **Drug Susceptibility Testing**

506 Minimum inhibitory concentrations (MICs) were determined by broth microdilution in 96-
507 well plates. Bacterial suspensions were prepared by washing log-phase cultures and
508 resuspending in Tween-80-free 7H9 medium to OD_{600} 0.01. Equal volumes (100 µL) of
509 bacterial suspension and 2× serial drug dilutions were combined and incubated at 37°C
510 for 6 days. Metabolic activity was assessed by adding 50 µL alamarBlue reagent (1:1

511 mixture of 10× alamarBlue and 10% Tween-80) and measuring fluorescence after 24
512 hours (544/590 nm excitation/emission) on a Fluostar OPTIMA plate reader (BMG
513 Labtech).

514 **Transmission Electron Microscopy**

515 Transmission electron microscopy was performed previously [27]. Briefly, mid-log phase
516 bacteria were collected and resuspended in 3% formaldehyde, 2.0% glutaraldehyde, 80
517 mM Sorenson's phosphate, and 4 mM MgCl2, pH 7.2 with duplicate samples for negative
518 staining and sectioning. Samples were fixed overnight at 4°C before processing and
519 imaging. Cell wall thickness was measured at two locations per bacterium: one in a thin
520 region and one in a thick region when present, to account for regional variations.

521 **Time-Kill Assays**

522 For nutrient-rich conditions, bacterial cultures were grown to mid-logarithmic phase in
523 complete 7H9 medium, then diluted to OD_{600} 0.05 one day prior to drug exposure. On
524 day 0, rifampin was added to a final concentration of 2 µg/mL unless otherwise noted
525 (stock: 2 mg/mL in DMSO), with vehicle controls receiving equivalent DMSO volumes.
526 Each condition was performed in triplicate using 5 mL cultures in 50 mL conical tubes,
527 incubated at 37°C with shaking. Bacterial viability was quantified by serial dilution plating
528 on 7H11 agar at days 2, 4, and 6 post-treatment. Plates were incubated at 37°C for 14-
529 21 days before colony counting.

530 For nutrient starvation conditions, log-phase cultures were washed and resuspended in
531 PBS containing 0.05% Tween-80 to OD_{600} 0.5, then incubated without shaking at 37°C
532 for 14 days to establish nutrient-deprivation. Rifampin (2 µg/mL) or vehicle control was
533 then added, and bacterial survival was monitored by CFU plating as described above.

534 For phosphate depletion experiments, cultures were washed twice and resuspended in
535 phosphate-free 7H9 medium to OD_{600} 0.05. Growth and survival were monitored over
536 time by CFU enumeration, with samples taken at indicated intervals during incubation at
537 37°C with shaking.

538 **Macrophage Infection Assays**

539 THP-1 cells were differentiated by treatment with 50 ng/mL phorbol 12-myristate 13-
540 acetate (PMA) for 48 hours at 37°C with 5% CO_2. Differentiated macrophages were
541 optionally activated with 20 ng/mL recombinant IFN-γ for 24 hours before infection.
542 Bacterial suspensions were prepared from log-phase cultures and used to infect
543 macrophages at a multiplicity of infection (MOI) of 1:1. After 4 hours, extracellular bacteria
544 were removed by washing three times with PBS, and fresh medium was added. At
545 specified time points, macrophages were lysed with 0.05% SDS and lysates were plated
546 on 7H11 agar for CFU determination.

547 **Polyphosphate Quantification**

548 Polyphosphate levels were measured as described previously [41]. Bacterial pellets were
549 lysed in guanidine thiocyanate buffer (5 M guanidine thiocyanate, 50 mM Tris-HCl pH 7.0)
550 using bead beating (3 cycles, 20 seconds at 6,800 rpm). After centrifugation, supernatants
551 were stored at -20°C, and protein concentrations were determined using DC Protein
552 Assay (Bio-Rad) for normalization. Polyphosphate was isolated using the GeneClean III
553 Kit (MP Biomedicals) following manufacturer protocols, including RNase/DNase
554 treatment to remove nucleic acids. Polyphosphate concentrations were determined
555 fluorometrically using DAPI staining (100 µg/mL final concentration) with
556 excitation/emission at 415/525 nm.

557 **Transcriptomic Analysis**

558 Wild-type and Δ*pstA1* cultures were grown to mid-log phase (OD_{600} ~0.5) in 45 mL 7H9
559 medium. To capture early transcriptional responses, cultures were treated with
560 subinhibitory rifampin (7.5 ng/mL, 1/4 MIC) or vehicle. RNA samples were collected at
561 baseline, 30- or 60-minutes post-treatment with three independent biological replicates
562 per condition.

563 Bacterial pellets (15 mL culture) were resuspended in TRIzol with 0.1 mm zirconia beads
564 and lysed using a Precellys Evolution homogenizer (3 cycles, 30 seconds at 7,400 rpm).

565 RNA was extracted with chloroform, precipitated with ethanol, and purified on RNeasy
566 columns (Qiagen) with on-column DNase I digestion. Following polyA selection, strand-
567 specific libraries were prepared using NEBNext Ultra II Directional RNA Library Prep Kit
568 and sequenced on Illumina NovaSeq X Plus (2×150 bp paired-end) at the Johns Hopkins
569 Genetic Resources Core Facility.

570 Raw reads were quality-assessed (FastQC v0.12.1) [30] and aligned to the *M.*
571 *tuberculosis* H37Rv reference genome (Bowtie2 v2.5.1) [31]. Gene counts were
572 quantified with featureCounts (Subread v2.0.0) [32]. Differential expression analysis used
573 DESeq2 v1.44.0 with significance thresholds of adjusted p-value < 0.05 (Benjamini-
574 Hochberg) and $|\log_2 FC| > 1$. Pathway analysis was performed using KEGGREST to query
575 KEGG pathways [34] for significantly differentially expressed genes.

576 **RT-qPCR Validation**

577 Twelve genes were analyzed by RT-qPCR, including eleven target genes and sigA as a
578 housekeeping control (18-22 nt, 100-147 bp amplicons) designed with IDT PrimerQuest
579 (Supplementary Table S1). Total RNA (500 ng) was DNase-treated, purified on RNeasy
580 columns, and reverse transcribed with iScript RT Master Mix (Bio-Rad) alongside no-RT
581 controls. qPCR used 10 µL reactions with SYBR Green Supermix on a QuantStudio 3
582 system (95°C/10 min; 40 cycles: 95°C/15s, 60°C/1 min). Relative expression was
583 calculated using the ΔΔCt method with sigA normalization.

584 **Metabolite extraction and LC-MS analysis**

585 *Mtb*-laden filters were incubated on m7H10 plates at 37°C for 5 days to mid-log phase
586 [35]. Cells were quenched with prechilled acetonitrile:methanol:H_2O (40:40:20, v:v:v) at
587 −40°C. Metabolites were extracted through mechanical lysis using 0.1-mm zirconia beads
588 in a Precellys tissue homogenizer for 2 min at 6,000 rpm, repeated three times under
589 continuous cooling at ≤2°C. Cell lysates were clarified by filtration through 0.22-µm Spin-
590 X columns. Residual protein content was measured with a BCA protein assay kit (Thermo
591 Scientific) to normalize metabolite ion counts to cell biomass.

592 Extracted metabolites were separated using a Cogent Diamond Hydride Type C column
593 with mobile phases of solvent A (ddH_2O with 0.2% formic acid) and solvent B (acetonitrile
594 with 0.2% formic acid). An Agilent 6230 TOF mass spectrometer was coupled to an
595 Agilent 1290 LC system. Dynamic mass axis calibration was maintained via continuous
596 infusion of reference mass solution through an isocratic pump with a 100:1 splitter. This
597 setup achieved mass errors of ~5 ppm and mass resolution of 10,000-25,000 over m/z
598 range 62–966, with a dynamic range of 5 $\log_{10}$. Ions were identified based on unique
599 accurate mass and retention time. Data analysis used Agilent Profinder B.07.00 with
600 mass tolerance <0.005 Da. All metabolomics data represent the average of at least two
601 independent triplicates.

602 **Antibiotic permeability (rifampin uptake)**

603 Rifampin uptake was measured using a previously validated device that enables timed
604 start-stop measurements of antibiotics in spent media [35, 36]. The device comprises a
605 plastic inset containing m7H9 with known antibiotic concentration. *Mtb*-laden filters were
606 generated using wild-type, ΔpstA1, and complement strains and placed onto media
607 containing 1 µg/mL rifampin at 37°C. After incubating for 2, 6, and 24 hours, bacterial
608 lysates and spent m7H9 were analyzed for rifampin content by LC-MS. Rifampin uptake
609 was calculated as: [RIF]drug only – [RIF]filtrate, with three biological replicates.

610 **Statistical Analysis**

611 Data are presented as mean ± standard deviation unless otherwise noted. Statistical
612 comparisons were performed using appropriate tests as specified in figure legends
613 (unpaired t-tests, one-way or two-way ANOVA with post-hoc corrections). Multiple
614 comparisons were corrected using the Holm-Sidak method or Tukey's Multiple
615 Comparisons where applicable. Statistical significance was defined as $p < 0.05$. Sample
616 sizes are indicated in individual figure legends.

617

618

Code and Data Availability

Code to replicate the performed analysis is available at https://doi.org/10.5281/zenodo.17495175. Raw sequencing data will be deposited to the Gene Expression Omnibus (GEO) once database submissions resume. Additional data supporting the findings of this study are available upon request.

Acknowledgements:

We thank Dr. Kenan Murphy (University of Massachusetts) for generously providing the pKM444 and pKM464 plasmids used for ORBIT cloning, and Dr. Gyanu Lamichhane (Johns Hopkins University) for providing the pMH94 episomal vector. We are grateful to Megan Gelement (Johns Hopkins University) for contributions to methodology and data analysis discussions. We are grateful to SeqCenter (Pittsburgh, PA) for whole genome sequencing services. We thank the Johns Hopkins Genetic Resources Core Facility (GRCF) for RNA sequencing services.

Funding:

These studies were supported by NIAID/NIH grants R21 AI167027 to PCK ; R01AI168088 to HE. The funding source had no role in the study design, data collection, data analysis, data interpretation or writing of the report. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions:

Conceptualization: CD, PCK; Methodology: CD, GVAC, JSB, GYL, HE, PCK; Data acquisition: CD, GVAC, GYL; Data analysis: CD, GVAC, GYL; Supervision: JSB, HE, PCK; Funding acquisition: PCK, HE; Writing of original draft: CD, GVAC, PCK; Writing—review & editing: CD, GVAC, JSB, GYL, HE, PCK

644 **Competing Interests:**

645 All authors declare that they have no competing interests.

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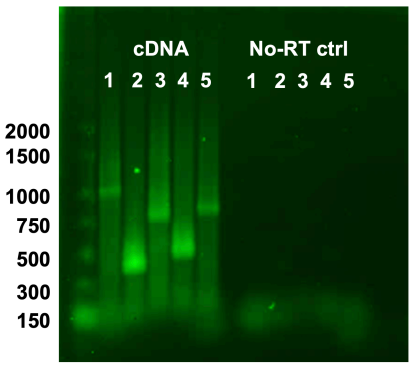
764

765 **Figures**

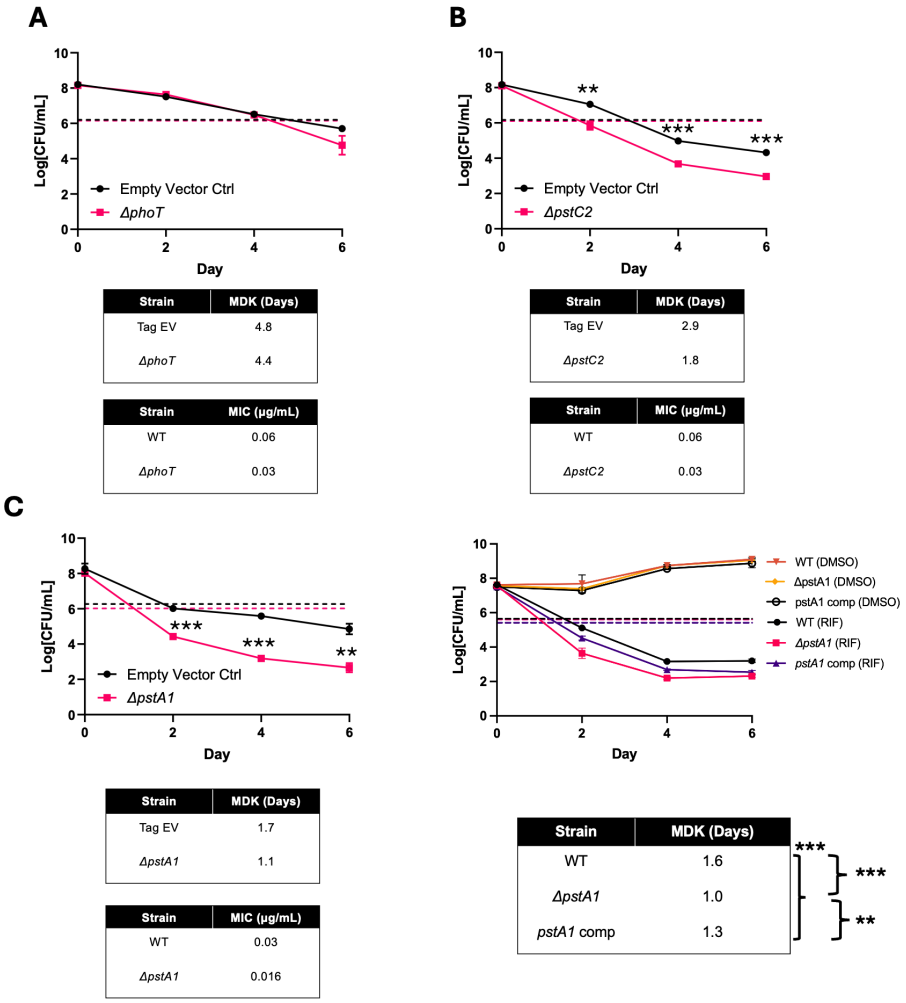


Lane	Region Amplified	Band Size
1	pstS3	1028 bp
2	pstS3-pstC2 junction	414 bp
3	pstC2	755 bp
4	pstC2-pstA1 junction	483 bp
5	pstA1	810 bp

766

767 **Figure 1. The *pstS3-pstC2-pstA1* genes are co-transcribed as an operon.** RT-PCR
768 analysis of H37Rv total RNA using primers spanning intragenic regions within *pstS3* (lane
769 1), *pstC2* (lane 3), and *pstA1* (lane 5), or intergenic junctions between *pstS3-pstC2* (lane
770 2) and *pstC2-pstA1* (lane 4). Amplification of all regions from cDNA confirms continuous
771 transcription across the three genes. No-RT controls (right panel) show the absence of
772 genomic DNA contamination. Expected amplicon sizes are indicated in the table.

773



Strain	MDK (Days)
Tag EV	4.8
ΔphoT	4.4

Strain	MIC (μg/mL)
WT	0.06
ΔphoT	0.03

Strain	MDK (Days)
Tag EV	2.9
ΔpstC2	1.8

Strain	MIC (μg/mL)
WT	0.06
ΔpstC2	0.03

Strain	MDK (Days)
Tag EV	1.7
ΔpstA1	1.1

Strain	MIC (μg/mL)
WT	0.03
ΔpstA1	0.016

Strain	MDK (Days)
WT	1.6
ΔpstA1	1.0
pstA1 comp	1.3

774

775 **Figure 2. Deletion of phosphate-specific transport genes enhances rifampin**
776 **susceptibility.** Time-kill assays were performed in 7H9 medium with 2 μg/mL rifampin for
777 (A) ΔphoT, (B) ΔpstC2, and (C) ΔpstA1 in parallel with an empty vector control. (D) Time-
778 kill assays performed with 7H9 medium with 0.5 μg/mL rifampin on wild-type, ΔpstA1 and
779 pstA1 complement strains. Dotted lines indicate 2-$\log_{10}$ reduction from starting inoculum.
780 Tables show minimum duration of killing (MDK) to achieve 2-$\log_{10}$ reduction and rifampin
781 MIC values for each strain. Statistical significance for CFU comparisons at individual
782 timepoints (A-C) was determined by unpaired t-test with Holm-Šídák correction for
783 multiple comparisons. Statistical significance for MDK values (D) was determined by one-
784 way ANOVA with Tukey's multiple comparisons test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

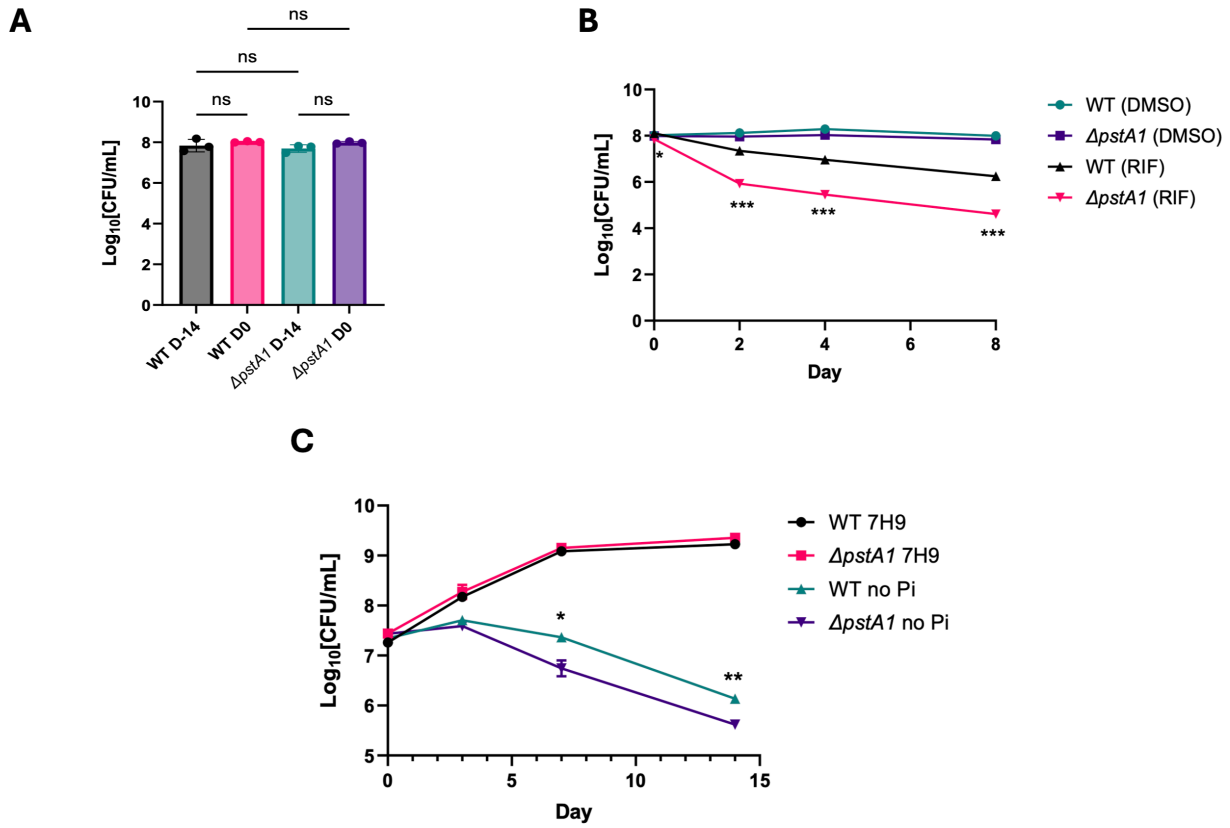


Figure 3. Δ*pstA1* survival under nutrient starvation and phosphate depletion. (**A**) Wild-type H37Rv and Δ*pstA1* survival after exposure to PBS-Tween for 14 days. No significant differences were observed by one-way ANOVA followed by Tukey's multiple comparisons test. (**B**) Δ*pstA1* maintained increased rifampin sensitivity after 2 weeks of PBS pre-treatment. Bacteria were treated with 2 µg/mL rifampin or DMSO vehicle control. Statistical differences indicated are for WT (RIF) vs Δ*pstA1* (RIF). Representative of two independent experiments.

(**C**) Δ*pstA1* shows no growth defect in phosphate-rich 7H9 medium but exhibits accelerated death under phosphate depletion (no Pi). Statistical differences shown are between WT no Pi and Δ*pstA1* no Pi conditions. Representative of three independent experiments.

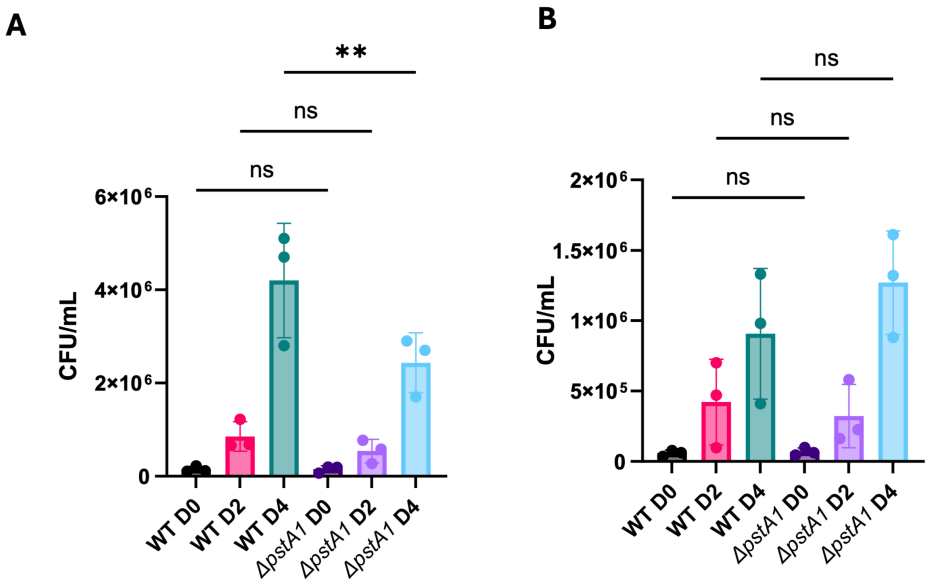


Figure 4. Δ*pstA1* exhibits an IFN-γ-dependent growth defect within macrophages. Bacterial burden within PMA-differentiated THP1 cells after 4 hours of infection (D0) or 2 or 4 days without (A) or with (B) 24 hr IFN-γ polarization before infection. One way ANOVA was performed followed by Sidak's multiple comparisons test to determine significance. Adjusted p- value ≤0.05 = *; ≤0.01 = **; ≤0.001 = ***.

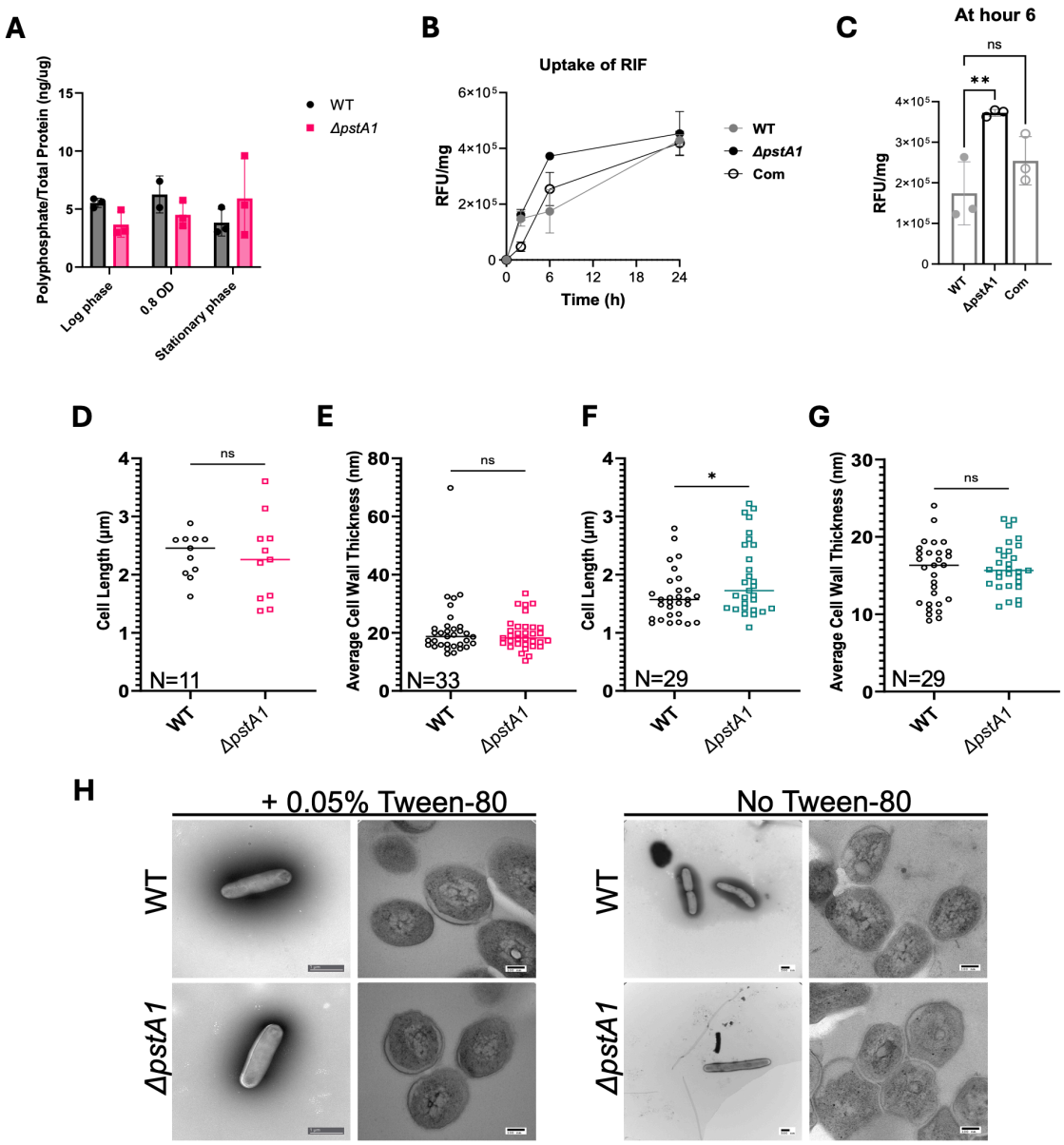


Figure 5. Rifampin uptake kinetics and cell morphology analysis of Δ*pstA1*. (**A**) Intracellular polyphosphate levels across different growth phases for wild-type and Δ*pstA1* normalized to total protein concentration. No significant differences were observed by one-way ANOVA followed by Tukey's multiple comparisons test. (**B-C**) Rifampin uptake kinetics in wild-type, Δ*pstA1*, and *pstA1* complement strains measured in a filter culture system. Bacteria were treated with 1.0 μg/mL rifampin and uptake was calculated as (initial rifampin concentration in media at 0 h - rifampin concentration at each timepoint) normalized to protein concentration. (**D-E**) Cell length and average cell

813 wall thickness of wild-type and Δ*pstA1* cultures grown in 7H9 medium with 0.05% Tween-
814 80 during log phase. (**F-G**) Cell length and average cell wall thickness of cultures grown
815 without Tween-80. Cell length measurements: n=11 bacteria per strain (with Tween-80),
816 n=29 bacteria per strain (without Tween-80). Cell wall thickness was determined by
817 averaging two measurements per bacterium: n=33 bacteria per strain (with Tween-80),
818 n=29 bacteria per strain (without Tween-80). (**H**) Representative transmission electron
819 microscopy images of whole bacteria or thin sections from log-phase cultures grown with
820 or without 0.05% Tween-80. Data represent mean ± SD. Statistical significance was
821 determined by one-way ANOVA followed by Tukey's multiple comparisons test (**B-C**) or
822 unpaired t-test (**D-G**). $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; ns, not significant.

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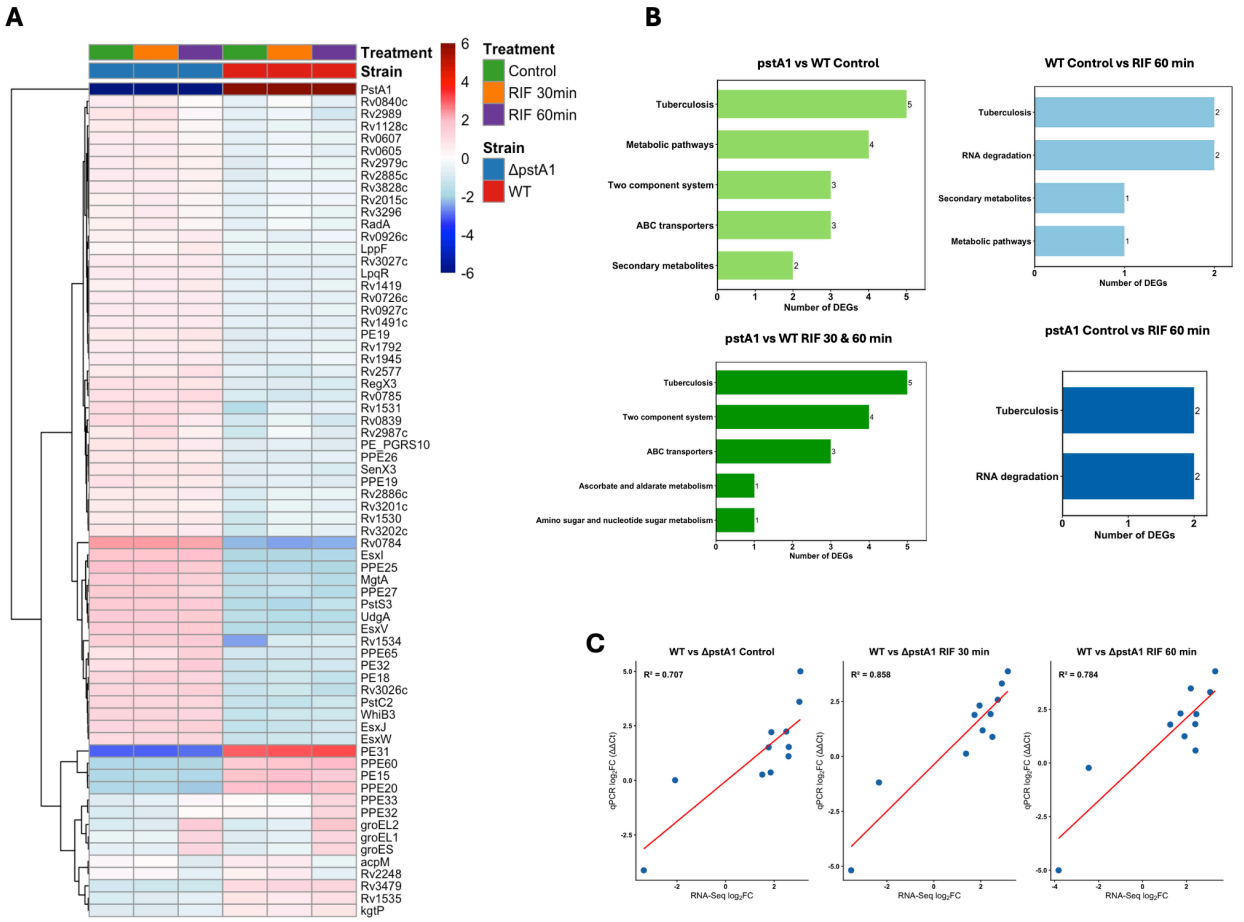
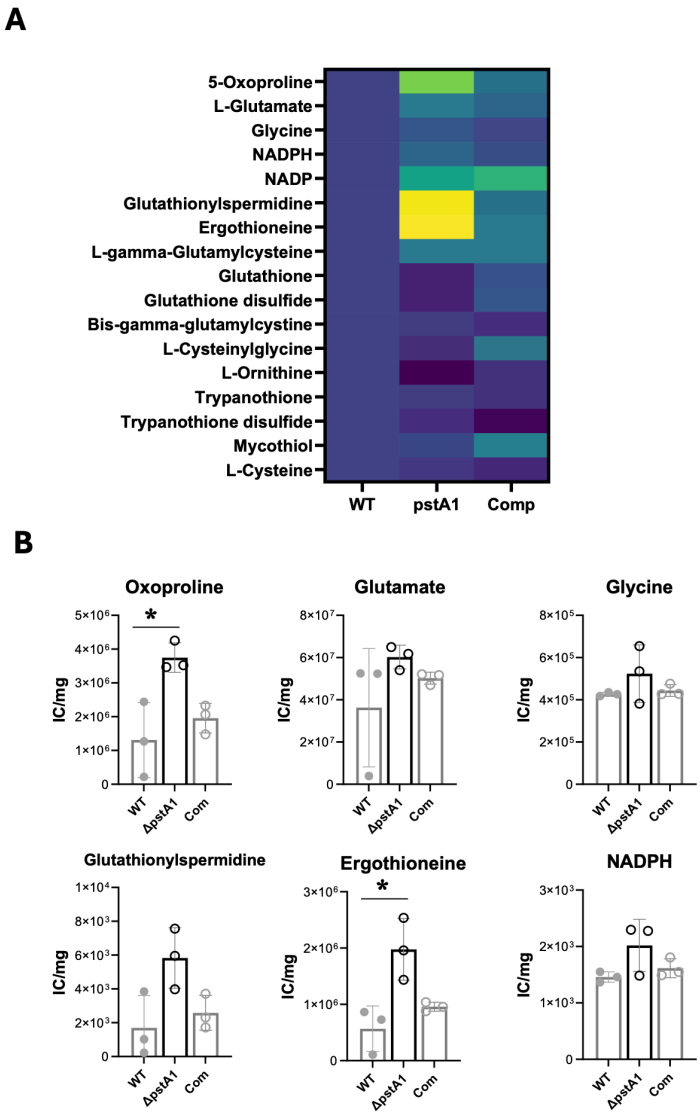


Figure 6. Transcriptional profiling of Δ*pstA1* with and without rifampin treatment. (**A**) Heatmap of differentially expressed genes (DEGs) in wild-type and Δ*pstA1* strains at baseline and after rifampin treatment (30 and 60 min). Genes were filtered for absolute $\log_2$ fold change ≥ 1 and adjusted $P < 0.05$. Each row represents a gene, and each column represents a biological replicate. Color scale indicates $\log_2$ fold change relative to wild-type control. (**B**) Functional enrichment analysis of DEGs for key comparisons: Δ*pstA1* vs wild-type control (top left), wild-type control vs wild-type with 60 min rifampin treatment (top right), Δ*pstA1* control vs Δ*pstA1* with rifampin treatment at 30 and 60 min (bottom left), and wild-type control vs Δ*pstA1* with 60 min rifampin treatment (bottom right). Bar plots show the number of DEGs in each functional category. Correlation between RNA-seq and RT-qPCR validation for selected genes across three comparisons. Each point represents a gene, with correlation coefficients (R^2) indicated for each comparison.



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841 **Figure 7. Metabolomic profiling reveals elevated antioxidant metabolites in**
842 **rifampin-treated Δ*pstA1*.** (**A**) Heatmap showing relative abundances of intracellular
843 metabolites involved in antioxidant and redox pathways in wild type, Δ*pstA1*, and *pstA1*
844 complement strains after 6 hours of rifampin exposure. Color scale indicates relative
845 metabolite abundance (normalized to internal standards and protein concentration). (**B**)
846 Quantification of selected antioxidant pathway metabolites. Bars represent mean ± SEM
847 from three biological replicates. Statistical significance was determined by one-way
848 ANOVA followed by Tukey's multiple comparisons test ($P < 0.1$).

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Supplementary Figures

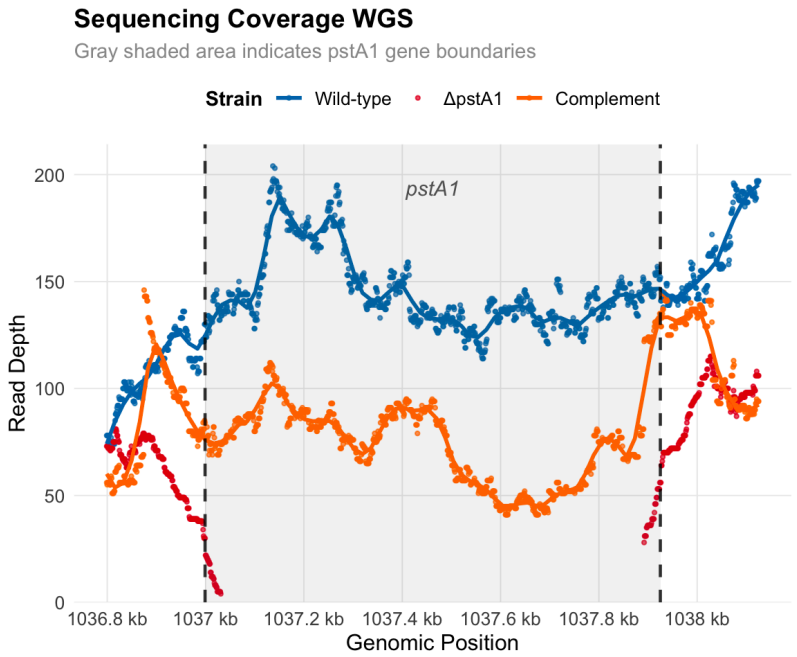

Figure S1. Whole genome sequencing of pstA1 deletion and complementation strains.

Read depth coverage across the *pstA1* locus in wild-type H37Rv (blue), ΔpstA1 deletion mutant (red), and Δ*pstA1::pstA1* complement strain (orange). Gray shaded region indicates pstA1 gene boundaries (coordinates 1,036,999-1,037,925 bp). Vertical dashed lines mark gene start and end positions. Wild-type exhibits uniform coverage (~150-200× read depth) throughout the *pstA1* coding sequence. The Δ*pstA1* strain shows near-zero coverage within the deleted region with maintained coverage in flanking sequences, confirming successful targeted deletion. The complement strain displays reduced coverage (~50-100× read depth) within *pstA1* relative to wild-type, consistent with episomal expression from the pMH94 plasmid. Individual points represent sequencing depth at each nucleotide position; smoothed trend lines (LOESS, span = 0.1) are shown for wild-type and *pstA1* complement strains. Coverage was calculated from reads aligned to the *M. tuberculosis* H37Rv reference genome using samtools depth and visualized in R.

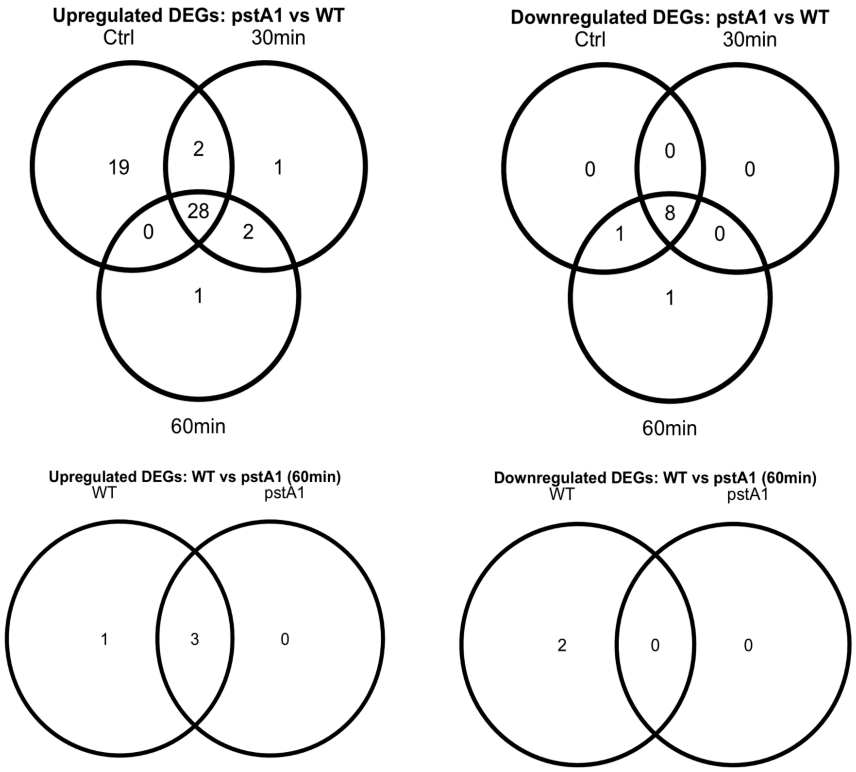

Figure S2. Venn diagram analysis of differentially expressed genes between Δ*pstA1* and wild-type strains. Overlap of differentially expressed genes (DEGs) identified by RNA-seq (log2 fold change > 1, adjusted p-value < 0.05). Top panels show upregulated (left) and downregulated (right) genes in Δ*pstA1* compared to WT across control, 30 min, and 60 min rifampin treatment conditions. Bottom panels show upregulated (left) and downregulated (right) genes comparing WT and Δ*pstA1* responses to 60 min rifampin treatment relative to their respective untreated controls. Numbers indicate the count of unique or shared DEGs in each category.

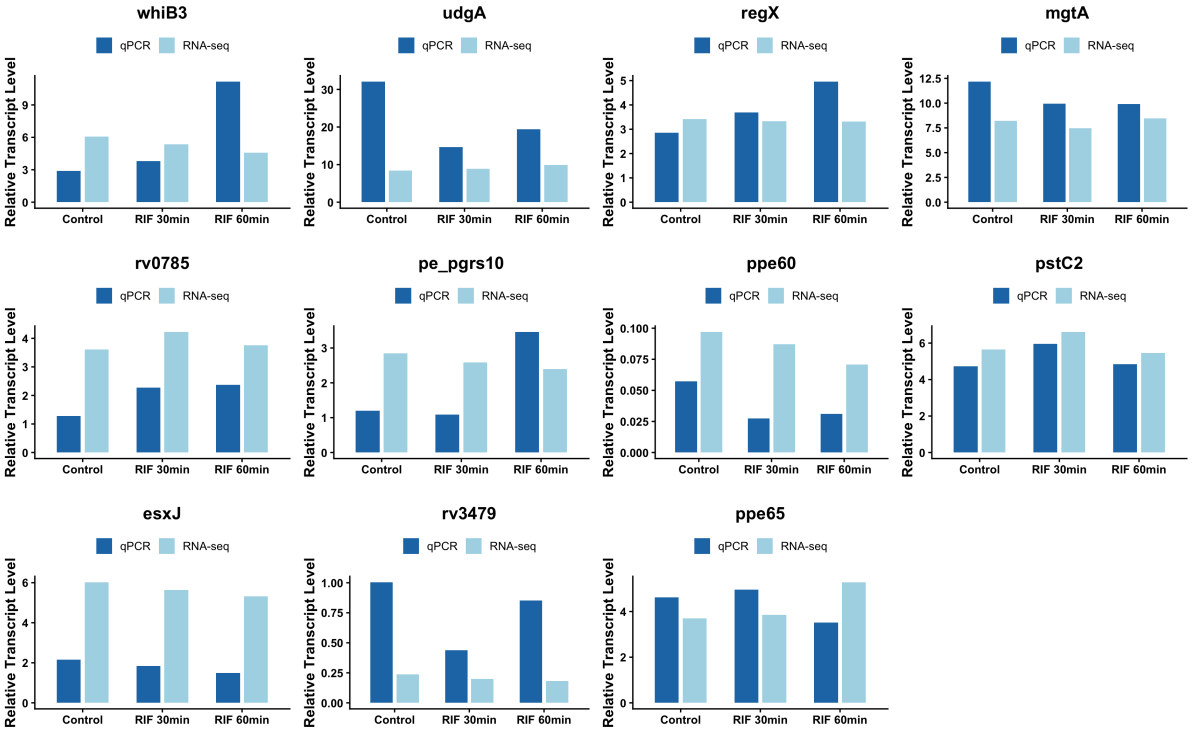

Figure S3. Validation of RNA-seq results by quantitative PCR. Comparison of transcript levels measured by RNA-seq (light blue) and qPCR (dark blue) for selected differentially expressed genes in wild-type *M. tuberculosis* under control conditions and following 30 or 60 min rifampin treatment. Genes were selected to represent diverse functional categories including transcriptional regulators (whiB3, regX), metabolic enzymes (udgA, mgtA), PE/PPE family proteins (pe_pgrs10, ppe60, ppe65, rv3479), phosphate transport (pstC2), and ESX secretion (esxJ). Relative transcript levels are normalized to control conditions. Strong correlation between RNA-seq and qPCR measurements validates the RNA-seq dataset.

897 **Supplementary Table S1. Oligonucleotide primers used for RT-qPCR analysis of**
898 ***Mycobacterium tuberculosis* genes**

Target Gene	Direction	Sequence (5′→3′)	Length (bp)	GC Content (%)	Melting Temperature (°C)	Amplicon Size (bp)
sigA	Forward	CGACGAAGACCACGAAGAC	19	57.9	62	131
sigA	Reverse	CGTCTTCATCCCAGACGAAAT	21	47.6	62	
pstA1	Forward	CGTTGGTCCTGCTGATGTT	19	52.6	62	111
pstA1	Reverse	TCCATTTCGGAACGCCTAAC	20	50.0	62	
whiB3	Forward	ACTCATCGATGTTCTTCCATCC	22	45.5	62	147
whiB3	Reverse	AAACGCCATAGGGCTCAC	18	55.6	62	
udgA	Forward	GTCCACGTGTACGATCCAAAG	21	52.4	63	123
udgA	Reverse	CCATTCGGTAAGCACCAACA	20	50.0	63	
regX	Forward	CTACGTGACCAAGCCCTATTC	21	52.4	62	132
regX	Reverse	TCCACATCCATGCGAACC	18	55.6	62	
mgtA	Forward	CACCTTCCGAATGCTGAGA	19	52.6	62	145
mgtA	Reverse	CGGAACATCGGTTTGGTAGA	20	50.0	62	
rv0785	Forward	GGCACGGTATCCGGATTATT	20	50.0	62	132
rv0785	Reverse	CATGTCCAGACTTGGTGATGTA	22	45.5	62	
pe_pgrs10	Forward	CAACACGACGGGATTGTTGA	20	50.0	63	131
pe_pgrs10	Reverse	GCACGAACTGGGCGTAAA	18	55.6	63	
ppe60	Forward	TCAGCAACGTCAGTTCGATAG	21	47.6	62	100
ppe60	Reverse	GGAGCTAAGCCCTTCAACAT	20	50.0	62	
pstC2	Forward	GGCGAATCACGCCAATTTC	19	52.6	62	117
pstC2	Reverse	AGAGCCGTTATCGAACTCAAC	21	47.6	62	
esxJ	Forward	GACACCATGACCCAGATGAAT	21	47.6	62	108
esxJ	Reverse	CTGCTCTTGCTGTTCGTAGT	20	50.0	62	
rv3479	Forward	TCCGATCTCAGTGTGCTAGT	20	50.0	62	112
rv3479	Reverse	CCGTGGATTGTTGGAGATAGTC	22	50.0	62	
ppe65	Forward	GCAGTTGTCGCCATTCAATC	20	50.0	62	144
ppe65	Reverse	CTAAGCCCAAACAACGCTTTAG	22	45.5	62	

899 **Abbreviations:** bp, base pairs; GC, guanine-cytosine; RT-qPCR, reverse transcription
900 quantitative polymerase chain reaction.

All primers were designed to amplify *Mycobacterium tuberculosis* target genes. Primer pairs generate amplicons ranging from 100 to 147 bp in length.

Supplementary Table S2. All genes meeting the significance threshold (|log2 fold change| > 1, adjusted p-value < 0.05) across seven pairwise comparisons are listed. Comparisons include temporal responses to rifampin treatment in wild-type and Δ*pstA1* strains (30 and 60 min versus untreated controls), and strain-specific differences between Δ*pstA1* and wild-type under control and rifampin-treated conditions. For each gene, the following information is provided: comparison name, *M. tuberculosis* H37Rv gene identifier (Rv number), gene name, log2 fold change, adjusted p-value (Benjamini-Hochberg correction), and KEGG pathway annotations where available. A total of 58 genes were differentially expressed between Δ*pstA1* and wild-type under control conditions (49 upregulated, 9 downregulated), 41 at 30 min rifampin (33 upregulated, 8 downregulated), and 41 at 60 min rifampin (31 upregulated, 10 downregulated). Only 6 genes responded to rifampin in wild-type (4 upregulated, 2 downregulated at 60 min), and 3 genes in Δ*pstA1* (all upregulated at 60 min), highlighting the substantial transcriptional differences between the strains.