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REVIEWS

Review

Acid Fasting: Modulation of *Mycobacterium tuberculosis* Metabolism at Acidic pH

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Mycobacterium tuberculosis **(Mtb) senses and adapts to acidic host environments during the course of pathogenesis. Mutants defective in acidic pH-dependent adaptations are often attenuated during macrophage or animal infections, supporting that these pathways are essential for pathogenesis and represent important new targets for drug discovery. This review examines a confluence of findings supporting that Mtb has restricted metabolism at acidic pH that results in the slowing of bacterial growth and changes in redox homeostasis. It is proposed that induction of the PhoPR regulon and anaplerotic metabolism, in concert with the restricted use of specific carbon sources, functions to counter reductive stress associated with acidic pH.**

Highlights

Mtb mutations that impair acidic pH-dependent adaptations often result in attenuation in macrophage and animal infections, supporting that pH-dependent adaptations are required for pathogenesis.

Mtb regulates gene expression in response to acidic pH via the PhoPR two-component regulatory system. These adaptations result in increased anabolism of cell-envelope lipids that may help alleviate reductive stress encountered at acidic pH.

Mtb slows or arrests its growth at acidic pH. Slowed and arrested growth is associated with induction of the PhoPR regulon and genes associated with anaplerotic metabolism.

Provided specific carbon sources or mutations, Mtb does not arrest or slow its growth at acidic pH, supporting that slowed growth is a genetically controlled adaptation. Acid fasting is proposed as a mechanism employed by Mtb to counter metabolic stress.

Acidic pH and *Mycobacterium tuberculosis* Pathogenesis

A growing body of research supports that adaptation to acidic pH is important to *Mycobacterium tuberculosis* (Mtb) pathogenesis. Mtb colonizes environments of varying acidity, including the phagolysosome and the **granuloma** (see Glossary). These environments can range from the mildly acidic **mycobacterial phagosome** (~pH 6.4) to the more strongly acidic environment of the lysosome (pH 4.5) [1,2]. The pH of the environment is dynamic and can fluctuate in different environments, depending on macrophage activation status [2,3], or natural variation in granulomas (ranging from pH 5.0 to 7.2, with a median pH of 5.5) [4]. Additionally, should Mtb perforate the phagosome [5], it would experience a more neutral pH of the cytoplasm (pH 7.2) or, in hypercapnic lung microenvironments, the pH may be more acidic. Thus, changes in pH may act as a direct stress that Mtb must adapt to, but it may also serve as a cue regarding the host environment to direct Mtb to regulate growth, metabolism, and energetics to optimize its physiology for a specific niche.

Several Mtb virulence factors have been associated with pH-dependent adaptations. The OmpATb membrane protein is required for optimal growth at acidic pH and virulence in mice [6] and functions to promote ammonia secretion and neutralization of the surrounding environment (Table 1) [7]. Additionally, Vandal *et al.* [8–10] identified multiple Mtb mutants with enhanced sensitivity to acidic pH *in vitro*, and one of these mutants, the *marP* serine protease, cannot maintain intrabacterial pH homeostasis and has reduced survival in activated macrophages and mice [8,10]. MarP has been further studied in the context of the *Mycobacterium marinum*/zebrafish infection model and shown to be required for *M. marinum* survival within the phagolysosome, a highly acidic environment [11]. These virulence defects support the premise that Mtb acid resistance is required *in vivo* and that pH-dependent pathways may function as new drug targets.

The importance of pH-dependent adaptations in Mtb is also evident by examining its transcriptional response during infection. Rohde *et al.* [12] observed the induction of 68 Mtb genes 2 hours after infection; when acidification of the phagosome is blocked with the vacuolar ATPase inhibitor concanamycin A, 30 of these genes are no longer induced. Such transcriptional adaptation to acidic pH also appears to be important to Mtb pathogenesis. For example, the acid- and phagosome-induced *phoPR* regulon is required for Mtb virulence in macrophages and animal models of tuberculosis (TB) infection [13–15]. Although PhoPR is required for optimal growth at pH 4.5 *in vitro* [16], the *phoPR* mutant grows well under the mildly acidic conditions associated with the macrophage phagosome (pH 6.5–5.5) [17,18]. At these mildly acidic pH conditions *in vitro* or in macrophages, the PhoPR regulon is strongly induced [12,17,19,20], suggesting that, at acidic pH, Mtb undergoes adaptations beyond those simply promoting acid resistance.

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Mtb regulates many genes involved in metabolic adaptation in response to acidic pH, including induction of genes involved in lipid metabolism and anaplerosis [12,17,20], suggesting that this is an important aspect of acid adaptation in Mtb. Additionally, several studies have also observed slowed growth of Mtb in response to acidic pH [17,20,21]. This pH-dependent adaptation is unique from that of closely related nonpathogenic *Mycobacterium* strains [21]. The intracellular pH of Mtb remains neutral even when exposed to conditions as acidic as pH 4.5 [8,22], revealing that slowed growth of Mtb is not due to a loss in cytoplasmic pH homeostasis. This review discusses the mechanisms of metabolic adaptation at acidic pH in the context of Mtb physiology and pathogenesis. A synthesis of the reviewed studies supports a model where acidic pH promotes Mtb to remodel and restrict its metabolism, and in some conditions, limit its use of exogenous carbon sources to counter **reductive stress**. This altered physiology is associated with slowed growth, nonreplicating persistence, pathogenesis, and drug tolerance, supporting the importance of pH-dependent adaptations when considering the development of new TB therapies.

Acidic pH Regulates Mtb Growth, Signaling, and Redox Homeostasis

Mtb growth is slowed at acidic pH in several different media. In a rich medium containing a variety of carbon sources (e.g., 7H9 medium containing glucose, glycerol, and oleic acid) Mtb slows its growth at acidic pH, with slowed growth beginning below pH 6.5 and complete growth arrest occurring at pH 5.0 [20]. Piddington *et al*. [21] observed that Mtb fully arrests its growth in defined Sauton's medium (containing glycerol, glucose, and albumin as carbon sources) at pH 6.0 and low Mg^{2+} levels (10 μM), whereas at higher Mg^{2+} levels (100 μM) Mtb exhibits slowed growth compared with that at pH 7.0. The dependence of Mtb on magnesium at acidic pH may reflect the importance of magnesium as a cofactor for metabolic enzymes or the role of the cation in maintaining cell envelope integrity, both factors that are modulated by acidic pH.

To further understand the mechanisms of Mtb growth restriction at acidic pH, a logical step is to consider its extensive transcriptional response, and central to this response is the PhoPR regulon. The PhoPR regulon is controlled by a two-component system consisting of the sensor histidine kinase PhoR and the response regulator PhoP [14]. Many genes regulated by acidic pH are induced immediately following Mtb phagocytosis by macrophages and remain induced during 2 weeks of macrophage infection and infection of mice [12,18–20]. A subset of these genes is regulated by PhoPR and controls virulence factors central to Mtb macrophage pathogenesis, including the ESX-1 secretion system [18,23] and the generation of cell-envelope lipids such as sulfolipid and acylated trehaloses [17,18,24], representing a significant shift in anabolic metabolism at acidic pH. The secretion of ESX-1 substrate, ESAT-6, is dependent on PhoPR [23] and can function to perforate phagosomal membranes [5,25], an activity that could neutralize the phagosome and alter available carbon sources, from the fatty acids and cholesterol of the phagosome to glycolytic carbon sources in the cytoplasm. Additionally, sulfolipid and acylated trehaloses have been shown to be required for normal arrest of phagosome maturation [26]; expression of these lipids is PhoPR- and acidic pH-dependent [18,24]. Thus, early in macrophage infection, induction of PhoPR can impact the pH of the phagosome and available carbon sources, supporting a potential physiological link between pH, carbon source, and macrophage pathogenesis.

In rich medium at acidic pH, Mtb also induces several genes encoding proteins involved in redox homeostasis, including thioredoxins, alkyl hydroperoxidase reductases, and the regulatory protein WhiB3. WhiB3 is regulated by PhoPR [27] and regulates the synthesis of lipids such as sulfolipid, poly- and diacyltrehalose, and phthiocerol dimycocerosate (PDIM) [28]. This WhiB3-regulated lipid synthesis acts as a reductive sink necessary to maintain redox homeostasis during hypoxia [28] and macrophage infection [29]. These data are consistent with Mtb experiencing reductive stress at acidic pH. Reductive stress is a phenomenon where a cell cannot replenish oxidized cofactors (such as NAD^+/$NADP^+$) and NADH/NADPH overaccumulate [30]. Paradoxically, cells undergoing reductive stress can have enhanced oxidative stress, as metabolic adaptations required to oxidize NADH/NADPH can result in the formation of reactive oxygen species (ROS). Alternative routes to overcome reductive stress include lipid anabolism, which results in the oxidation of NADPH.

Glossary

Acid fasting: an adaptation that promotes the use of specific carbon sources and remodeling of metabolism to actively limit growth at acidic pH. This physiology is proposed to counter metabolic stress encountered at acidic pH.
Acid growth arrest: a model of Mtb nonreplicating persistence, where the bacterium is grown at pH 5.7 with glycerol as the sole carbon source [60]. Mtb remains viable, metabolically active, and becomes drug tolerant. Mutations in *ppe51* promote growth under these conditions, supporting that acid growth arrest is an adaptive physiology.
Anaplerotic node: the branch of metabolism that links flux between glycolysis, gluconeogenesis, and the TCA cycle [36]. Enzymes of this node promote metabolism of PEP, pyruvate, and oxaloacetate.
Granuloma: a localized inflammatory structure composed of macrophages and lymphocytes that can form at the site of infection.
Mycobacterial phagosome: the vacuole that forms around Mtb phagocytosed by macrophages. The phagosome is acidified by a vacuolar ATPase and has a pH varying from pH 6.4 to 5.5, depending on the activation status of the macrophage. Mtb can perforate the phagosome, which could neutralize the vacuole.
PEP-glyoxylate cycle: a metabolic pathway employing glyoxylate shunt (*icl*) and gluconeogenic (*pckA*) enzymes that promotes metabolism while limiting production of reduced cofactors and CO_2 release by the oxidative branch of the TCA cycle [55].
PPE proteins: a family of mycobacterial proteins that contain an N terminal domain enriched in proline-proline-glutamate (PPE). These proteins, many of which may be exported, have been associated with modulating the host immune response and nutrient acquisition.
Reductive stress: a condition where cells overaccumulate reduced cofactors (such as NADH/NADPH) and metabolism is limited [30]. Reductive stress can be mitigated by lipid anabolism or oxidation of cofactors, a process that requires specific anaplerotic metabolites or can generate reactive oxygen species.

Mtb gene	Name	Gene induced at acidic pH[a]	*phoP*-dependent induction[b]	Mutant phenotypes associated with acidic pH adaptation and virulence	Refs
Rv0211	*pckA*	Yes	No[c]	Reduced growth at pH 5.7 in rich medium; attenuated in mice	[39,60]
Rv0467	*icl1*	Yes	No	Reduced growth at pH 5.7 in rich medium; attenuated in mice	[17,44,45,60]
Rv0757	*phoP*	Yes	Yes[c]	Enhanced growth at pH 5.7 in minimal medium with pyruvate; reduced growth in rich medium at pH 4.5; reduced synthesis of sulfolipid, acylated trehaloses; TAG accumulation, reduced esat-6 export. Attenuated in mice and guinea pigs	[13,15–18,20,23,24]
Rv0899	*ompATb*	No	No	Reduced growth at acidic pH 5.5 and in macrophages; reduced ammonia secretion to neutralize acidic pH; attenuated in mice	[6,7]
Rv1161, Rv1162	*narGH*	Yes	No	Reduced survival at pH 5.5 and hypoxia	[34]
Rv1221	*sigE*	Yes	No	Reduced expression of PhoP and acidic pH induced genes; attenuated in mice	[16,80]
Rv2395a	*aprA/mcr7*	Yes	Yes[c]	Enhanced TAG accumulation, reduced Twin Arginine Translocation secretion; reduced survival in macrophages	[20,81]
Rv3136	*ppe51*	Yes	Yes	Enhanced growth with glycerol as a sole carbon source at pH 5.7; reduced drug tolerance	[60]
Rv3416	*whiB3*	Yes	Yes[c]	Reduced synthesis of sulfolipid and acylated trehaloses; altered redox homeostasis at acidic pH; increased ergothioneine production; reduced survival in macrophages; attenuated in guinea pigs	[27–29,78,82]
Rv3671c	*marP*	No	No	Loss of cytoplasmic pH homeostasis *in vitro* and macrophages; loss of processing of RipA peptidoglycan hydrolase leading to cell elongation; attenuated in mice	[10,83]
Rv3825c	*pks2*	Yes	Yes[c]	Reduced accumulation of sulfolipid; altered phagosome maturation in combination with mutants in pks3/4 and PDIM	[26,84]
Rv3875	*esat-6*	Yes	Yes	Reduced perforation of the phagosome membrane; attenuated in mice	[5,25,85]

Table 1. Selected Genes Associated with Mtb pH-Dependent Adaptation and Pathogenesis

[a]Induction at acidic pH determined based on published transcriptional profiles examining Mtb under various acidic conditions *in vitro* [12,17,18,20,86].

[b]PhoP regulation determined based on altered gene expression in a *phoP* mutant [22,24,31,79].

[c]PhoP promoter binding evidence with Chip-seq or other functional assay [27,82,87–89].

Notably, using an RoGFP reporter, Mtb has been shown to have a reduced cytoplasmic potential at acidic pH, and in a PhoPR mutant, the cytoplasmic potential is further reduced [17]. Additionally, enhanced ROS and sensitivity to thiol-oxidative stress have been observed in Mtb at acidic pH as compared with neutral pH [31]. It is possible that enzymes with NADH oxidase activity, such as KatG [32], may contribute to the enhanced accumulation of ROS observed at acidic pH [31]. Low-molecular-weight thiol buffers, such as mycothiol and ergothioneine, could also function to mitigate reductive stress. Mycothiol pools have been shown to increase twofold during acid stress (pH 5.5) [33], further supporting adaptation to shifts in redox poise at acidic pH. Thus, like hypoxia, acidic pH appears to promote reductive stress, and a network of responses controlled by PhoPR, WhiB3, NADH oxidases, and low-molecular-weight thiols may play a role in mitigating reductive stress (Figure 1).

Why Mtb induces genes involved in redox homeostasis at acidic pH has not been fully explained, but one clue comes from the remodeling of the electron transport chain. At acidic pH, genes encoding type-I NADH dehydrogenase are repressed transcriptionally, whereas those encoding type II dehydrogenase and *bd*-type cytochrome oxidases are induced [17] (Figure 1). This change in

respiration machinery constitutes a shift from proton-translocating to nonproton-translocating components. Whether this shift is in response to an increased extracellular proton concentration or is responsible for changes in Mtb redox poise remains to be investigated. Further evidence of restricted respiration at acidic pH comes from the observation that exogenous nitrate as an alternative terminal electron acceptor enhances Mtb viability at acidic pH and hypoxia, and mutations in nitrate reductase result in significantly impaired survival [34]. Additionally, a small molecule inhibitor of Mtb respiration, C10, promotes killing of Mtb at pH 5.5 in Sauton's medium, but has limited activity at pH 7.0 [35]. Based on these findings, we propose a model in which PhoPR and WhiB3 may be induced at acidic pH to help alleviate reductive stress associated with limited respiration, by promoting the synthesis of cell envelope lipids, which would function to oxidize reduced cofactors such as NADPH (Figure 1).

Carbon Source-Dependent Regulation of Growth at Acidic pH

Given the evidence of pH playing a role in the regulation of metabolic and redox genes, Mtb growth was studied in minimal medium in several different carbon sources, in media strongly

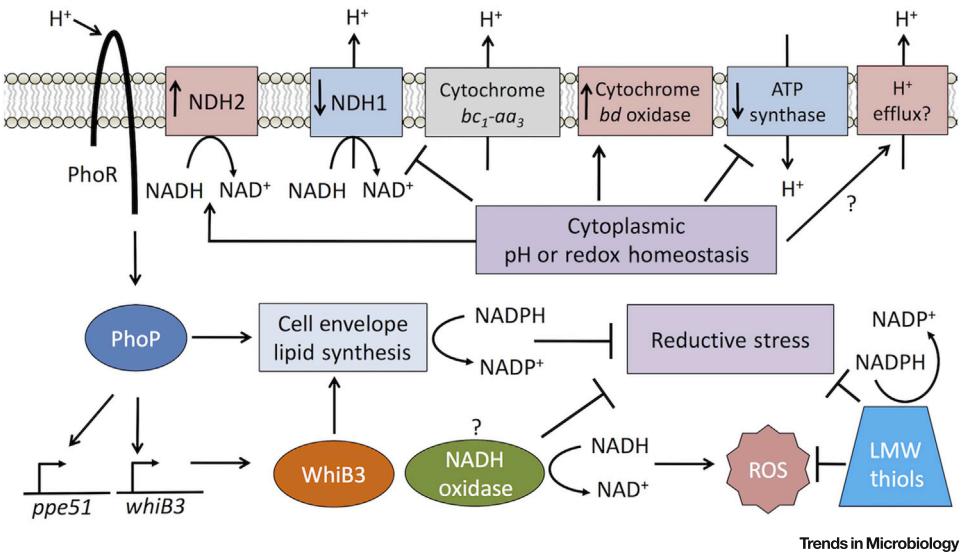

Figure 1. *Mycobacterium tuberculosis* (Mtb) Signaling and Redox Homeostasis Adaptations at Acidic pH. Acidic pH promotes transcriptional and physiological adaptations that are associated with changes in intracellular redox homeostasis. This model is a synthesis of the observed adaptations, and it presents a speculative framework for how these changes promote pH-dependent adaptations. The PhoPR regulon is induced by acidic pH and induces the redox sensor *whiB3* and genes for synthesis of acylated trehaloses (e.g., *pks2* and *pks3*) which promote anabolism of cell-envelope lipids [17,18,24,27]. Mutations in *phoP* and *whiB3* are associated with reductive stress, supporting that this lipid synthesis may mitigate reductive stress by oxidizing NADPH [17,28,29]. Reductive stress may also be mitigated by direct oxidation of NADH by NADH oxidases, such as KatG, which will generate reactive oxygen species (ROS) [32]. KatG is induced at acidic pH [17], and enhanced intracellular ROS have been observed at acidic pH [31], suggesting that ROS accumulation may be associated with reductive stress. Low-molecular-weight (LMW) thiols, such as mycothiol or ergothioneine, could function to mitigate this ROS stress and also directly mitigate reductive stress by consuming NADPH [29,78,79]. Transcriptional changes in the respiratory chain (shown as up or down arrows) are observed at acidic pH, supporting remodeling of respiration at acidic pH [17]. It is hypothesized that electron transport is altered at acidic pH due to the need to maintain cytoplasmic pH homeostasis by efflux of protons. In support of this hypothesis, proton-translocating type I NADH dehydrogenase genes are repressed, while the nonproton-translocating type II enzyme is induced [17]. These adaptations could enable oxidation of NADH without further increasing proton motive force. Downregulation of ATP synthase genes [17] at acidic pH could function to slow growth and limit proton translocation into the cytoplasm. These combined adaptations may function to replenish pools of oxidized cofactors and maintain redox and pH homeostasis.

buffered at pH 7.0 or pH 5.7. Mtb growth at acidic pH required host-associated carbon sources that function at the intersection of glycolysis and the tricarboxylic acid cycle (TCA) cycle, such as phosphoenolpyruvate (PEP), pyruvate, acetate, oxaloacetate (OA), and cholesterol [17]. In contrast, in other tested carbon sources, Mtb fully arrests its growth at acidic pH and establishes a state of non-replicating persistence (Figure 2). Growth-arrested Mtb is resuscitated by the addition of pyruvate, suggesting that growth arrest is due to a pH-dependent checkpoint on metabolism or nutrient acquisition. Therefore, carbon sources that feed the PEP–pyruvate–OA **anaplerotic node** [36] promote growth at acidic pH. Notably, the nonpathogen *Mycobacterium smegmatis* grew similarly well at acidic and neutral pH on all tested carbon sources, supporting that this physiology is an adaptation associated with pathogenesis.

Mtb begins to slow its growth in minimal medium with glycerol at pH 6.4, the same pH threshold at which the *phoPR* regulon is induced by acidic pH [20], suggesting that *phoPR* may play a role in carbon-source-dependent growth arrest. To explore this hypothesis, the response of a CDC1551 *ΔphoPR* deletion mutant [37] was examined during growth on single carbon sources at acidic pH. At pH 5.7, the *phoP* mutant, like the wild type (WT), maintained arrested growth on glycerol; however, the *phoP* mutant exhibited significantly enhanced growth on pyruvate when compared with WT Mtb. This finding shows that *phoPR* slows growth at acidic pH and, thus, potentially explains why reduced growth and induction of the *phoPR* regulon are associated [20]. It is possible that PhoPR slows growth at acidic pH by diverting carbon away from the TCA cycle to lipid anabolism. However, the metabolic plasticity provided by pyruvate may enable Mtb to efficiently anabolize lipids, while also fueling central carbon metabolism. This mechanism is similar to enhanced growth observed by a triacylglycerol synthase (*tgs1)* mutant during hypoxia [38]. Hypoxia induces the *tgs1* gene through the DosRST two-component regulatory system, promotes anabolism of triacylglycerol (TAG), and diverts acetyl-CoA away from the TCA cycle. In both hypoxia and acidic pH, Mtb experiences reductive stress, supporting that inducing lipid anabolism, by DosRST or PhoPR, represents a shared mechanism to

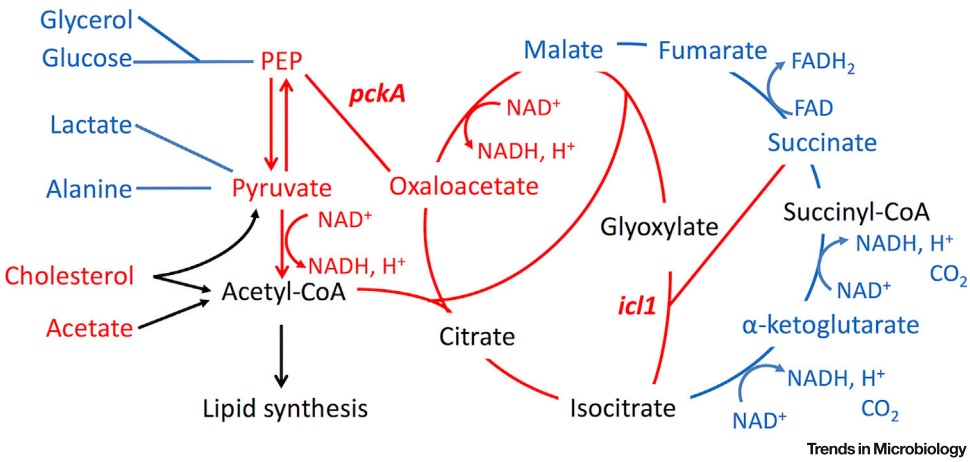

Figure 2. *Mycobacterium tuberculosis* (Mtb) Restricts its Metabolism at Acidic pH.
At acidic pH, Mtb exhibits arrested growth on various single carbon sources (shown in blue) that support growth at neutral pH [17]. In contrast, carbon sources that feed the anaplerotic node (shown in red) promote growth at both acidic and neutral pH. Notably, *icl* and *pckA*, genes that encode enzymes that promote anaplerotic metabolism, are strongly induced at acidic pH (in both permissive and nonpermissive conditions). Mutations in these genes cause reduced growth at acidic pH in rich medium and altered accumulation of metabolites in defined media [60]. Together, these data suggest that, at acidic pH, Mtb may shift its metabolism to the phosphoenolpyruvate (PEP)-glyoxylate pathway (highlighted in red), which would limit metabolism around the oxidative branch of the tricarboxylic acid cycle (TCA) cycle and decrease synthesis of NADH and CO_2 release. These changes would directly mitigate reductive stress and conserve carbon, which can then be used to synthesize lipids or other metabolites to further mitigate reductive stress. Notably, mutations in *ppe51* enable growth on glycerol at acidic pH, suggesting that the bacterium is adapted to limit metabolism at acidic pH [60].

regenerate oxidized cofactors. Therefore, modulation of metabolism by hypoxia and acidic pH may function to make additional metabolites available for lipid anabolism and maintenance of redox homeostasis.

Metabolic Adaptations at Acidic pH

Metabolic adaptations to environmental stresses will initially be broadly discussed, to place pH-dependent adaptations into context. During infection, the metabolic requirements of Mtb differ from those encountered *in vitro*, as evidenced by the number of central carbon metabolism enzymes that are required specifically *in vivo*. Although dispensable for growth in a nutrient-rich medium *in vitro*, phosphoenolpyruvate carboxykinase (encoded by *pckA*) [39,40], lipoamide dehydrogenase (encoded by *lpdC*) [41], dihydrolipoamide acyltransferase (encoded by *dlaT*) [42], the E1 subunit of α-ketoglutarate dehydrogenase (encoded by *hoas*) [43], and the bifunctional methylisocitrate/isocitrate lyase (encoded by *icl*) [44] are required for full virulence during mouse infection. Further characterization of these enzymes has helped uncover the metabolic environments encountered during infection that lead to their role in pathogenesis. For example, *pckA* is required for gluconeogenic carbon flow when grown on fatty acids, a somewhat surprising finding given that Mtb contains genes that are annotated as encoding malic enzyme (*mez*), pyruvate carboxylase (*pca*), and pyruvate phosphate dikinase (*ppdK*), suggesting that, despite these alternative routes of metabolism, Mtb specifically requires *pckA* for gluconeogenesis during infection [39]. Additionally, *icl* is required for the metabolism of propionyl-CoA generated from the catabolism of cholesterol, methyl-branched fatty acids, and odd-chain fatty acids [45–47], carbon sources utilized during Mtb growth *in vivo* [48–51]. As can be seen from these examples, probing the *in vivo* requirements for metabolic enzymes and pathways has provided a better understanding of the metabolic constraints incurred by the host environment.

Understanding Mtb metabolism during infection has also been probed by culturing Mtb *in vitro* in media with host-mimicking environments or stresses and measuring the adaptive metabolic response. The findings observed in this approach have complemented the studies of *in vivo* essentiality, and, in the case of isocitrate lyase, expanded the observed functions of this enzyme in promoting Mtb survival. Under conditions of *in vitro* hypoxia, Mtb uses the reductive TCA cycle to maintain membrane potential in a process that leads to succinate accumulation in the medium [52,53]. The glyoxylate shunt also appears to be an important metabolic adaptation to hypoxia, as Eoh and Rhee [53] observed that the *icl* mutant has reduced succinate secretion and survival under hypoxia, defects that can be restored by the addition of the reductive TCA precursor aspartic acid [53]. This hypoxia-induced metabolic remodeling appears to be dependent on the absence of oxygen as an electron acceptor, because addition of the alternative electron acceptor nitrate limits succinate secretion by Mtb [53]. Thus, increased metabolism via the reductive TCA cycle, and specifically the enzyme *icl*, are implicated in Mtb's metabolic adaptation to hypoxic environments.

The anaplerotic genes *pckA* and *icl1* are strongly induced by acidic pH in both glycerol and pyruvate, with enhanced induction in pyruvate. Muñoz-Elías and McKinney initially suggested that *pckA* and *icl1*, which are both essential for Mtb virulence in mice [39,44,45], may be required to promote metabolism via the **PEP-glyoxylate cycle** [54]. Fisher and Sauer showed that slow-growing, glucose-limited *Escherichia coli* in continuous culture completely oxidized glucose via a metabolic cycle that they named the PEP-glyoxylate cycle, which requires flux through the glyoxylate shunt [55]. Notably, this cycle was active in an NADPH-overproducing mutant of *E. coli*, which led the authors to speculate that the purpose of this PEP-glyoxylate cycle was to decouple central carbon catabolism from NADPH production that occurs through the oxidative TCA cycle. The PEP-glyoxylate cycle would enable the full oxidation of carbon sources that fuel the anaplerotic node [55], such as pyruvate or acetyl-CoA. During Mtb infection, these carbon sources are physiologically relevant as they are the products of cholesterol or fatty acid catabolism, carbon sources Mtb has been shown to metabolize in macrophages [49,56–58]. Notably, storage lipids in foamy macrophages are enriched for TAG, supporting that, when metabolized, fatty acids, and glycerol may be important carbon sources in

infected macrophages *in vivo* [59]. In this manner, the PEP-glyoxylate cycle may promote efficient energy-producing catabolism while keeping PEP, pyruvate, OA, and acetyl-CoA abundant and available for anabolism. Together, these data support a model in which acidic pH: (i) induces PhoP-dependent lipid anabolism to oxidize redox cofactors and slow growth by diverting carbon from central metabolism, and (ii) drives Mtb physiology to remodel its metabolism, perhaps by adopting the PEP-glyoxylate cycle, to balance energy production and carbon utilization. This modulated metabolism would help to mitigate reductive stress by limiting NADH produced by the oxidative TCA cycle, while limiting CO_2 production, such that carbon is available for lipid anabolism to oxidize NADPH (Figure 2).

Based on this model, it is predicted that *icl* or *pckA* would be required for optimal growth of Mtb at acidic pH. Indeed, in CDC1551 (which has two *icl* genes, *icl1/2*), deletion of *icl1/2* or *pckA* results in reduced growth at acidic pH when compared with the WT in rich medium, but do not impact growth at neutral pH [60]. Additionally, the enhanced growth of the *phoP* mutant on pyruvate at acidic pH is inhibited by the Icl inhibitor 3-nitropropionic acid [17]. Metabolomics studies showed that growth at acidic pH with pyruvate as the sole carbon source causes a decrease of succinyl-CoA accumulation and enhanced malate accumulation and succinate secretion, consistent with a model in which acidic pH promotes flux through the glyoxylate shunt and decreases flux through the oxidative TCA cycle [60]. Deletion of *pckA* further enhances malate accumulation and succinate secretion, supporting that, concomitant with *icl* induction, *pckA* induction at acidic pH functions to divert carbon away from the TCA cycle. Thus, transcriptional profiling, genetic deletion, and metabolomics studies provide supporting evidence that Mtb remodels metabolism at the anaplerotic node in response to acidic pH (Figure 2).

Acidic pH and Carbon Source-Dependent Growth Arrest

During nonreplicating persistence, Mtb exhibits markedly improved tolerance to several chemically distinct antibiotics [38,61–63], supporting the hypothesis that growth rate is an important determinant of drug efficacy during treatment of tuberculosis. One approach to confront this drug tolerance is to impair the ability of Mtb to properly adapt to or maintain growth arrest. This approach appears to hold promise, as the mutation of *tgs1* that resulted in increased replication in hypoxia and low iron conditions also led to increased susceptibility to isoniazid (INH), ethambutol, streptomycin, and ciprofloxacin both *in vitro* as well as in macrophage and mouse infection models [38]. Additionally, recently described inhibitors of DosRST, required for maintaining nonreplicating persistence in hypoxia, also decreased antibiotic tolerance to INH in Mtb cultured in hypoxia [64].

Metabolic restriction at acidic pH provides a new model of Mtb growth arrest. Mtb cultured at pH 5.7 in minimal medium containing glycerol as the single carbon source maintains viability and ATP pools in the absence of replication for the full duration of a 39-day experiment [60]. These results demonstrate that Mtb cultured under these conditions establishes a nonreplicating persistent state, a physiology that has been named **acid growth arrest**. To determine whether Mtb under acid growth arrest is still metabolically active, Mtb uptake of ^{14}C-glycerol was measured. Over time, Mtb accumulated ^{14}C-glycerol at pH 5.7, albeit ~70% lower than at pH 7.0, and radiolabel incorporation into trehalose di- and monomycolate, TAG, and sulfolipid was observed in growth-arrested Mtb. The uptake of glycerol, as well as its anabolic incorporation into Mtb lipids, suggests that Mtb under acid growth arrest is metabolically active, and supports the view that acid growth arrest is a metabolically active, nonreplicating state. During acid growth arrest, Mtb developed tolerance to INH, rifampicin (RIF), and SDS detergent stress.

In other persistent states of Mtb, such as starvation [65] or hypoxia [66], cessation of growth is understood to be due to a physiological limitation, such as absence of a carbon source or a terminal electron acceptor. However, Mtb under acid growth arrest is provided both a metabolically utilized carbon source, glycerol, as well as a terminal electron acceptor, oxygen. Therefore, instead of representing a physiological limitation, it is possible that acid growth arrest is a regulated adaptation

of Mtb. Indeed, in a genetic screen, mutants were isolated that could grow at pH 5.7 on glycerol as the sole carbon source. The *enhanced acid growth* (*eag*) mutants had single nucleotide variants that mapped to two distinct substitutions (S211R and A228D) in the proline-proline-glutamate (PPE) gene *ppe51* (also named MT3221). Overexpression of the PPE51-S211R variant protein in WT Mtb results in significantly enhanced acid growth, showing that the mutant allele is sufficient to promote a dominant gain-of-function, *eag* phenotype. Both *eag* mutants and PPE51-S211R overexpression strains exhibited >10-fold decreased tolerance to INH and RIF during acidic growth arrest [60]. These findings support that acid growth arrest is a genetically controlled, adaptive process and not simply a physiological limitation associated with acidic pH.

The *ppe51* gene is induced at acidic pH and in macrophages, and its expression is dependent on PhoP, supporting that its expression is associated with a carbon source and pH-dependent adaptation. Recent experimental evidence suggests a functional role in nutrient acquisition by PE/**PPE proteins**. In *M. marinum,* Ates and colleagues [67] showed that ESX-5, the *esx* gene cluster associated with most *pe/ppe* secretion [68], is involved in nutrient and/or metabolite acquisition. ESX-5 mutations in *M. marinum* result in significantly reduced growth on medium with Tween-40 or Tween-80 as the sole carbon source, and the ESX-5 mutant strain exhibits significantly impaired uptake of fluorescently labeled fatty acids compared with WT and complemented strains. These data support a role for ESX-5 in facilitating the uptake of fatty acids to be used as a carbon source. Furthermore, the ESX-5 mutant strain was deficient in secretion of 24 PE and PPE proteins, suggesting that they may play a role in ESX-5-dependent fatty acid and/or nutrient uptake. In further support of the ESX-5 substrate nutrient acquisition hypothesis, Mitra and colleagues showed that PPE36 and PPE62 proteins were surface exposed and required for heme utilization [69]. Together, these findings provide evidence that ESX-5 and PPE proteins can function in nutrient acquisition, possibly by acting like porins. The specific function of PPE51 remains to be determined, but its *eag* phenotype, and regulation by PhoP at acidic pH, provides support for the hypothesis that PPE51 may be induced and exported at acidic pH to modulate glycerol or other nutrient acquisition.

Adaptive Acid Fasting

Common themes and important distinctions exist between the growth regulation and metabolic adaptation of Mtb to acidic pH and the other environmental stresses discussed. Similar to other growth-arrest models, such as hypoxia or nitric oxide, acidic pH growth arrest does seem to involve perturbations in redox homeostasis as evidenced by measurement of intracellular redox poise as well as the transcriptional response of Mtb [17,31]. Unlike hypoxia or nitric oxide, neither oxygen limitation nor a direct inhibitor of respiration can easily account for the redox imbalance; however, the shift in electron transport chain machinery from proton pumping to nonproton-pumping could represent a response to a so far unexplored restriction in Mtb respiration at acidic pH. Unlike the starvation model of growth arrest, ample carbon source is available for Mtb catabolism during acid growth arrest. Given these differences, understanding the mechanisms of acid growth arrest may provide new insights into the constraints of Mtb growth *in vivo*.

It is worth noting that, unlike hypoxic growth arrest, where mutation leading to increased replication is detrimental to Mtb survival, the increased replication of the *phoPR* mutant does not reduce viability at acidic pH [16,17]. Similarly, *ppe51* mutants promote robust growth under conditions that normally induce nonreplicating persistence. These observations support that Mtb can grow well at pH 5.7, and that growth regulation at acidic pH is not due simply to physiological constraints incurred by acid stress. Rather, slowed growth may represent an adaptive process of Mtb. In this interpretation, the growth arrest observed at acidic pH is not starvation, an inability to utilize fuel for energy production and growth, but rather fasting, an active process of avoiding energy production for growth. Indeed, during acid growth arrest, Mtb radiolabeled with ^{14}C-acetate or ^{14}C-propionate has been shown to catabolize radiolabeled triacylglycerol and synthesize new radiolabeled sulfolipid and trehalose dimycolate [70]. Thus, as part of the **acid fasting** program, Mtb can rely on intracellular carbon sources as a metabolic buffer to promote metabolism required for survival. It remains to be determined if Mtb, preadapted to lack TAG stores, is able to establish and maintain NRP during acid growth arrest.

Outstanding Questions

What signals directly regulate PhoPR? The PhoPR regulon can be induced by acidic pH *in vitro* and in macrophages; however, it remains to be determined if PhoPR is directly responding to acidic pH. The PhoPR pathway is also induced by magnesium and chloride [14,37], and it is possible that changes in pH are altering the availability of extracellular signals, both *in vitro* or in macrophages. PhoPR induction can be inhibited with a carbonic anhydrase inhibitor, raising the possibility that PhoPR may be sensing CO_2 [18]. Alternatively, it is possible that PhoPR is responding to a nonconventional intrabacterial signal associated with altered physiology at acidic pH, such as changes in redox homeostasis or carbon metabolism [76]. Integrated genetic, biochemical, structural studies of the PhoR sensor kinase and its regulators will be required to further define direct mechanisms of PhoPR regulation.

Is Mtb dependence on anaplerotic metabolism *in vivo* driven by acidic environments? Icl and PckA are required for virulence during animal infections [39,44,45] and are also associated with optimal adaptation to acidic pH *in vitro* [17,60]. However, it remains to be determined if the attenuation phenotypes *in vivo* are associated with Mtb colonizing environments that are acidic, or perhaps due to changes in metabolism that are independent of acidic pH or also required in neutral environments. Studies examining survival of Mtb mutants at the single cell level in specific immune microenvironments in infected animals may provide a link between acidic pH and these enzymes *in vivo*.

What is the link between acidic pH, growth, and specific carbon sources *in vivo*? Depending on which niche Mtb is colonizing, it may be metabolizing different carbon sources. Use of Mtb mutants in metabolic or carbon-source uptake pathways may enable studies to

The acid fasting model assumes that factors necessary to maintain acid tolerance, such as MarP and OmpATb, are intact [6,7,10,11]; however, when acid tolerance is intact, the metabolic and growth restriction of Mtb at acidic pH may be better understood as a response to an environmental cue. Adaptation in response to acidic pH could prepare Mtb for the concomitant stresses encountered with acidic pH during infection, and as such it is tempting to speculate that Mtb has evolved to use the acidic pH of the phagolysosome as a cue for the forthcoming antimicrobial environment of the macrophage. Additionally, restricting growth on glycolytic carbon sources may be adaptive for survival in the macrophage (where lipids and cholesterol predominate), while, at the neutral pH of the cytoplasm, it may be advantageous to promote metabolism of glycolytic carbon sources available in the cytoplasm.

Concluding Remarks

Acidic pH-dependent adaptations are required for survival *in vitro*, nonreplicating persistence, drug tolerance, and virulence in macrophages and animals. Thus, acidic pH-dependent pathways represent important new targets that may function to shorten the course of TB therapy (see Outstanding Questions). Indeed, pyrazinamide (PZA) has enhanced activity at acidic pH, and its inclusion in first-line therapy has shortened the standard of care of TB therapy from 9 months to 6 months. Resistance to PZA has been associated with changes in coenzyme A and fatty acid metabolism [71,72], supporting the possibility that metabolic adaptations at acidic pH may be associated with PZA pH-dependent activity. Several compounds have been discovered that target pH-dependent physiologies, such as bacterial cytoplasmic pH homeostasis [73–75], PhoPR signaling [18], respiration [35], or reductive stress [31], and the early signs of efficacy of these compounds highlight the promise of targeting pH-dependent vulnerabilities. Defining these pH-dependent vulnerabilities has the potential to generate even more new therapies effective within this important environmental niche.

determine if pH is used as a cue to optimize metabolism for carbon sources associated with a specific niche.

Are metabolic adaptations at acidic pH associated with restricted respiration? It is possible that the mechanism required to maintain cytoplasmic pH homeostasis at acidic environments may interfere with membrane potential, ATP synthesis, and redox homeostasis. Further experiments, examining the requirement of specific electron transport chain genes and redox homeostasis genes (using inhibitors or knockdown by CRISPR interference [77]), will help to define specific mechanisms by which acidic pH modulates Mtb respiration and redox homeostasis.

What are common and specific features of nonreplicating persistence induced by different environments? Acidic pH, hypoxia, nitric oxide, and starvation can all drive Mtb to establish nonreplicating persistence and drug tolerance. Efforts to identify shared aspects of these persistent physiologies may help to define targets that can broadly impact persistence and drug tolerance.

Acknowledgments

We thank Andrew Olive for insightful feedback on this manuscript. This work was supported by funding from AgBioResearch and grants from the National Institutes of Health (NIH)-National Institute of Allergy and Infectious Diseases (NIAID) (U54AI057153 and R01AI116605).

Disclaimer Statement

R.B.A. is the founder and owner of Tarn Biosciences, Inc., a company that is working to develop new TB drugs.

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