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Epigenetic Phosphorylation Control of *Mycobacterium tuberculosis* Infection and Persistence

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ABSTRACT Reversible protein phosphorylation is the most common type of epigenetic posttranslational modification in living cells used as a major regulation mechanism of biological processes. The *Mycobacterium tuberculosis* genome encodes for 11 serine/threonine protein kinases that are responsible for sensing environmental signals to coordinate a cellular response to ensure the pathogen's infectivity, survival, and growth. To overcome killing mechanisms generated within the host during infection, *M. tuberculosis* enters a state of nonreplicating persistence that is characterized by arrested growth, limited metabolic activity and phenotypic resistance to antimycobacterial drugs. In this article we focus our attention on the role of *M. tuberculosis* serine/threonine protein kinases in sensing the host environment to coordinate the bacilli's physiology including growth, cell wall components, and central metabolism to establish a persistent infection.

PROTEIN PHOSPHORYLATION IN *MYCOBACTERIUM TUBERCULOSIS*

Protein phosphorylation is known to occur across all three kingdoms of life; however, the study of post-translational modification in bacteria was neglected for a considerable amount of time. Early attempts to detect its presence were unsuccessful, generating the dogma that protein phosphorylation was a regulatory mechanism that emerged late in evolution to meet the needs of organisms composed of multiple and differentiated cells. The pioneering work of several groups in the 1970s identified protein kinase activity in both *Escherichia coli* and *Salmonella typhimurium* (1–3), which soon led to the discovery of the histidine/aspartate kinases of the two-component systems (4, 5). The first aspect of this system involves the stimulation of a histidine kinase by a particular environmental or intracellular signal resulting in autophosphorylation on a key histidine residue. The phospho-histidine can then be used as a substrate by the cognate response regulator for its own autophosphorylation on an aspartate residue. The majority of response regulators are DNA binding proteins that trigger expression from target promoters. Unlike the cross-reactivity observed with serine/threonine/tyrosine (Ser/Thr/Tyr) kinases in eukaryotic cell signaling cascades, two-component systems work in isolation, where a given pairing of histidine kinase and response regulator are highly selective for each other via protein-protein interaction. Q1

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During the initial phase of the two-component system discovery, these systems were regarded as the major signal transduction pathway in bacteria, which led to the hypothesis that Ser/Thr/Tyr phosphorylation was a eukaryotic trait, whereas His/Asp phosphorylation was exclusive to prokaryotes. Since this time, our knowledge of protein phosphorylation has been revised. Hundreds of two-component systems have been discovered in eukaryotic cells (6), and recent genomic data indicate that "eukaryotic-like" Ser/Thr protein kinases (STPKs) are as prevalent in prokaryotes as in their histidine kinase counterparts (7). However, two-component systems remain the main signaling mechanism in all phyla of bacteria, with STPKs most abundant among *Acidobacteria*, *Actinobacteria* (including the genus *Mycobacterium*), various groups of *Cyanobacteria*, as well as bacteria belonging to the order of *Myxococcales*.

The pathogenic success of *M. tuberculosis* is largely dependent on its ability to sense and adapt to the dynamic environment of the host. As a result, *M. tuberculosis* has evolved an extensive intracellular signaling network consisting of 12 paired two-component regulatory systems (also including 4 orphan regulators), 11 STPKs, a single tyrosine kinase, and 3 phosphatases, which have been extensively reviewed in the past two decades (8–10). The presence of Ser/Thr and Tyr protein kinases, and two-component systems that phosphorylate substrates on Asp, enables the cell to generate phosphorylated residues with far greater stability. Generally, the hydrolytic half-time of phosphoryl-asp is only a couple of hours, whereas Ser/Thr/Tyr phosphor-esters, or *O*-phosphorylation, can produce signals that remain stable for weeks and require a phosphatase to be reversed (11). Consequently, *M. tuberculosis* uses phosphoryl-asp for rapid, short-term signal transduction and Ser/Thr/Tyr phosphorylation for long-term, global responses, giving cells the advantage to adapt and survive in complex environments.

The discovery of *M. tuberculosis* STPKs originated from the identification of 11 genes encoding for the subdomains of the Hank's superfamily of kinases, resulting in their annotation as "eukaryotic-like" STPKs (12). Yet it is possible that not all STPKs involved in *O*-phosphorylation have been identified due to our use of a eukaryotic-like biased paradigm. Of the 11 STPKs (PknA to PknL), the sequences of 9 contain a transmembrane region that connects the intracellular N-terminal kinase domain to a C-terminal sensory component located extracellularly. Current structural data indicate that these transmembrane receptor kinases are activated by dimerization of their kinase domains, resulting in the phosphorylation of the activation loop and ultimately leading to kinase activation (13). The remaining two kinases, PknK and PknG, lack a transmembrane domain. Yet subcellular fractions of *M. tuberculosis* lysates showed PknK to be present in the cell wall/membrane fraction rather than the cytosol through an unknown anchoring mechanism (14). Therefore, PknG is described as the sole soluble STPK in *M. tuberculosis*.

Due to the lack of Tyr kinases in the *M. tuberculosis* genome, Tyr phosphorylation was believed to be absent from *M. tuberculosis* despite the presence of two protein Tyr phosphatases: PtpA and PtpB (15). As a result, these protein phosphatases were originally hypothesized to be solely involved in the interference of host signaling pathways, which was shown by the ability of PtpA to inhibit host vesicular trafficking and phagosome acidification (16, 17). However, preliminary immunoblot evidence suggesting the existence of an *M. tuberculosis* protein phosphorylated on Tyr (18) led us to identify the first *M. tuberculosis* protein tyrosine kinase, PtkA, located within the same operon as its cognate substrate PtpA (19). Recent phosphoproteomic data has indeed found *M. tuberculosis* to support extensive Tyr phosphorylation (63 sites on 49 proteins) (20). Yet bioinformatic analysis has been unsuccessful in identifying any of the traditional bacterial tyrosine kinases (known as BY-kinases), suggesting that Tyr phosphorylation might be carried out strictly by a novel and "odd" family of Tyr kinases in *M. tuberculosis* (21). However, recent investigation by Kusebauch et al. (20) found that *M. tuberculosis* STPKs undergo Tyr phosphorylation in their activation segment, suggesting their action as dual-specificity (Ser/Thr/Tyr) kinases. Although plausible, this hypothesis has to be proven experimentally because none of the STPKs have been shown to phosphorylate their substrates on Tyr. On the other hand, the tyrosine kinase PtkA was shown to be Ser/Thr phosphorylated by and interact with several STPKs, (22) strengthening the idea of cross-phosphorylation between STPKs and Tyr kinases in *M. tuberculosis*.

Similar to other prokaryotes, the overall extent of *O*-phosphorylation in *M. tuberculosis* is limited, amounting to 7.5% of all proteins being phosphorylated (23), compared to the 40 to 45% of eukaryotes (24). As expected, the number of Ser/Thr phosphorylation sites in *M. tuberculosis* is significantly greater than that of Tyr, with over 500 sites identified (23). These findings indicate that each STPK can act on multiple substrates (Table 1). However, little is known regarding STPK signaling cascades and kinase hierarchy in *M. tuberculosis*. In contrast His-kinases, which typically phosphorylate a

single response regulator, the cross-reactivity observed with Ser/Thr/Tyr kinases results in complex signaling cascades. Rarely does the direct output of *M. tuberculosis* Ser/Thr phosphorylation involve the direct regulation of expression of target genes; however, some evidence exists of signaling organization typical of two-component systems (14, 25–27).

Recently, *in vitro* analysis of the interactions between all STPKs has added a novel layer of signaling in *M. tuberculosis* (28). Mapping of STPK phosphorylation suggests a three-layered architecture that includes master regulator (PknB and PknH), signal transducer (PknE and PknJ), and terminal substrate kinases (PknA, PknD, PknF, PknK, and PknL) as shown in Fig. 1. Master regulator kinases exclusively undergo autophosphorylation to achieve activation, which can in turn cross-phosphorylate downstream kinases. Signal transducing kinases can also autophosphorylate as well as cross-phosphorylate downstream kinases, which is likely to act as a mode to propagate signals to intracellular substrates. The remaining substrate kinases were unable to transfer phosphates to other STPKs, indicating that their molecular targets are limited to other protein substrates. Furthermore, four of these kinases (PknA, PknF, PknK, PknL) are thought to lack the machinery to detect extracellular signals and therefore rely on cross-phosphorylation by the upstream kinases for activation (28). Recent evidence showed PknA to autophosphorylate its own activation loop independent of PknB; however, the extracytoplasmic domain appears to be dispensable for PknA function (29). Unlike the other substrate kinases, PknD contains an extracellular β-propeller used by *M. tuberculosis* to sense osmotic stress and is also strategically positioned in the intracellular signaling network to regulate the "stressosome" in response to upstream STPKs (30–32). However, little is known about which ligands bind to these sensor domains or about what environmental stimuli they respond to (Table 2). Furthermore, it is evident that transcriptional data offer limited information about the environmental cues that are sensed by *M. tuberculosis* STPKs. Conditions have been reported where the transcriptional levels of STPKs have increased and meanwhile their protein levels remained unchanged (33). Furthermore, STPKs require activation through auto- or cross-phosphorylation prior to initiating any downstream signaling events. Therefore, it is recommended that caution be used when inferring relationships between environmental conditions and STPK function.

Our understanding of bacterial signal transduction has become very significant due to its role in *M. tuberculosis* pathogenicity. In recent years, STPKs have been shown to play a crucial role in the growth and survival of *M. tuberculosis* during infection. The intracellular cascades induced by STPKs culminate in alterations in gene transcription, enzymatic activity, cellular localization, and protein-protein interactions which translate into the rapid metabolic adaptation of the bacterium. Through our knowledge of their corresponding environmental stimuli and substrates, along with the bacilli's physiological responses, we dedicate this article to describing the role of STPKs in the growth and/or survival of *M. tuberculosis* to establish a persistent infection.

ESTABLISHING INFECTION THROUGH SUBVERSION OF INNATE IMMUNE RESPONSE BY STPKs

Following internalization by host macrophages, *M. tuberculosis* resides and replicates in intracellular membrane-bound vacuoles. Bacterial compartmentalization provides an enclosed space for the host cell to localize high concentrations of reactive oxygen species, reactive nitrogen intermediates, and enzymes that eliminate invading pathogens. Typically during infection, the phagosome fuses with endosomes and lysosomes, resulting in lumen acidification and the acquisition of proteolytic enzymes for lysosome-mediated degradation of invading microorganisms. The classical mechanism *M. tuberculosis* uses to evade the innate immune response is through inhibition of these cellular pathways, namely, phagosomal acidification and blockage of the fusion of phagosomes with lysosomes (34, 35). Therefore, sensing the intracellular environment of the macrophage, adapting its physiology, and responding to host defense mechanisms is an integral part of *M. tuberculosis* pathophysiology.

One of the fascinating strategies used by *M. tuberculosis* is its recently discovered ability to directly interfere with host signaling pathways. The best and first studied example of such interference is the utilization of the mycobacterial secreted Tyr phosphatase PtpA. PtpA was shown to possess phosphatase activity against the host vacuolar protein sorting 33B and glycogen synthase kinase-α, resulting in the arrest of phagosome maturation and prevention of macrophage apoptosis, respectively (16, 36). In addition, PtpA binds subunit H of the macrophage ATPase pump, resulting in blockage of phagosomal acidification and leading to subversion of one of the key characteristics of innate immunity (37, 38).

TABLE 1 Biochemically verified substrates of *M. tuberculosis* serine/threonine protein kinases

Kinase	Substrate function	Substrate
PknA (Rv0015c)	Cell division	FipA (179), FtsZ (85), ParB (180), Wag31 (66)
	Arabinan biosynthesis	EmbR (181)
	MA biosynthesis	KasA (101), KasB (101), FabD (101), FabH (102), HadAB/BC (99), InhA (97,98), MabA (96)
	PG biosynthesis	MurD (74)
	TCA cycle	Mdh (172)
	Methionine cycle	SahH (182)
	Signaling	PstP (183), PtkA (22)
	Protein chaperone	GroEL1 (184)
	Proteasome	PrcA (56)
	Hypothetical	Rv1422/CuvA (66)
PknB (Rv0014c)	Cell division	HupB (185), ParB (180)
	Arabinan biosynthesis	EmbR (181)
	MA biosynthesis	KasA (101), KasB (101), FabD (101), HadAB/BC (99), InhA (97, 98), MabA (96)
	PG biosynthesis	FhaA (186, 187), GlmU (67), MviN (68), PbpA (87), PonA1 (79)
	α-Glucan biosynthesis	GlgE (143)
	PDIM biosynthesis	PapA5 (115, 116)
	Erothioneine biosynthesis	EgtD (132)
	TCA cycle	GarA (40, 167, 188)
	Methionine cycle	SahH (182, 189)
	Signaling	PstP (183), PtpA (51)
	Protein chaperone	GroEL1 (184)
	Protein synthesis	EF-Tu (190)
	Proteasome	PrcA (56)
	Stress response	RshA (55), SigH (55)
	Hypothetical	Rv1422/CuvA (66), Rv0516c (30), Rv1747 (186)
PknD (Rv0931c)	Cell division	ParB (180)
	MA biosynthesis	FabD (101), FabH (102), HadAB/BC (99), KasA (101), KasB (101), MabA (96), PcaA (191)
	Ergothioneine biosynthesis	EgtD (132)
	TCA cycle	GarA (188), Mdh (172)
	Signaling	PtkA (22), PtpA (51)
	Protein chaperone	GroEL1 (184)
	Transport	MmpL7 (114), Rv1747 (186)
	Osmotic stress	OprA (30)
	Anti-antisigma factor?	Rv0516c (30)
PknE (Rv1743)	Cell division	HupB (185)
	Arabinan biosynthesis	EmbR (192), EmbR2 (192)
	MA biosynthesis	FabD (101), FabH (96), KasA (101), KasB (101), HadAB/BC (99), PcaA (191)
	PDIM biosynthesis	PapA5 (115)
	TCA cycle	GarA (188), Mdh (172)
	Methionine cycle	SahH (182)
	Signaling	PtpA (51)
	Protein chaperone	GroEL1 (184)
	Transport	Rv1747 (186)
	Stress response	RshA (30)
	Anti-antisigma factor	RsfA (30), Rv0516c (30), Rv1904 (30)
PknF (Rv1746)	Cell division	HupB (185), ParB (180)
	Arabinan biosynthesis	EmbR (192), EmbR2 (192)
	MA biosynthesis	FabD (101), FabH (96), KasA (101), KasB (101), HadAB/BC (99), InhA (98), PcaA (191)
	PG biosynthesis	FhaA (186)
	TCA cycle	GarA (188)
	Methionine cycle	SahH (182)
	Signaling	PtkA (22)
	Protein chaperone	GroE1 (184)
	Transport	Rv1747 (164)
PknG (Rv0410c)	TCA cycle	GarA (40, 167), Mdh (172)
	Oxidative stress/biofilm growth	L13 (193)

(continued)

TABLE 1 Biochemically verified substrates of *M. tuberculosis* serine/threonine protein kinases *(continued)*

Kinase	Substrate function	Substrate
PknH (Rv1266c)	Arabinan biosynthesis	EmbR (25, 194)
	MA biosynthesis	FadD (101), FabH (102), HadAB/BC (99), InhA (98), KasA (101), KasB (101), PcaA (191)
	PG biosynthesis	DacB1 (78)
	TCA cycle	Mdh (172)
	Methionine cycle	SahH (182)
	Signaling	PtpA (51)
	Protein chaperone	GroEL1 (184)
	Dormancy	DosR (27)
	Transcription	Rv0681 (78)
PknI (Rv2914c)	MA biosynthesis	FadD (101)
PknJ (Rv2088)	Arabinan biosynthesis	EmbR (195)
	MA biosynthesis	MmaA4 (195)
	TCA cycle	Mdh (172)
	Glycolysis	PykA (162, 165)
	Dipeptidase	PepE (195)
PknK (Rv3080c)	MA biosynthesis	FabD (196), VirS (14)
	Ergothioneine biosynthesis	EgtD (132)
	Signaling	PtkA (22)
PknL (Rv2176)	MA biosynthesis	FadD (101), InhA (97), KasA (101), KasB (101), MabA (96)
	Protein chaperone	GroEL1 (184)
	Methionine cycle	SahH (182)
	Signaling	PtpA (51)
	DNA binding?	Rv2175c (197)

Inhibition of Phagosome-Lysosome Fusion

M. tuberculosis STPKs are mainly localized to the mycobacterial membrane, and as such possess a limited role in the direct interaction with the host immune system. The only exception described has been that of PknG, which was shown to be secreted and suggested to directly phosphorylate host proteins (39). Although a direct host substrate was not yet identified, PknG was suggested to control phagosome-lysosome fusion of *Mycobacterium bovis* Bacille Calmette-Guérin (BCG)-containing phagosomes within macrophages (39), in contrast to *Mycobacterium smegmatis*, which was directly transferred to lysosomal compartments upon infection. However, PknG was shown to have a direct role in *M. tuberculosis* physiology (40). Since no host substrates or mechanism for PknG blockage of phagosome-lysosome fusion has been described, the effect on host response might be indirect and can be explained by the growth deficiency of the PknG mutant in macrophages and animal models (41).

Apoptosis

Consistent with *M. tuberculosis*'s ability to evade immune-mediated destruction, blockage of macrophage apoptosis upon infection enables bacterial persistence (42). PknE was shown to have a role in *M. tuberculosis* pathogenicity because the mutant demonstrated decreased survival due to increased host-apoptosis in both macrophage and murine models of infection (43, 44). In line with the role of nitric oxide (NO) in regulating the intrinsic apoptotic events of the cell (45), apoptosis occurred following treatment of the Δ*pknE* mutant with NO (43). Furthermore, *pknE* expression was shown to be upregulated in the presence of nitrate stress in *M. smegmatis*, although a polar effect of the mutation was not ruled out. Despite the study lacking a complement, these findings suggest that PknE senses NO inside the host and possibly interacts with other mycobacterial and/or host cell components, leading to inhibition of apoptosis.

The exact mechanism underlying the inhibition of apoptosis by PknE remains unknown; however, a role for PknE in modulating the expression of apoptotic proteins was suggested (46, 47). The *pknE* gene was shown to modulate the expression of toll-like receptors (TLRs), in agreement with their previously identified role in *M. tuberculosis* infections, by regulating apoptosis and inflammatory responses (48, 49). In addition, a number of PknE substrates are involved in regulating mycolic acid synthesis (Table 1). The uptake of mycolic acids has been previously shown to prolong the survival and increase Bcl-2 expression of the macrophage (50). Furthermore, mycolic acid extracted from *M. tuberculosis* was also shown to reduce the macrophage response to TLR2 agonists, which could prevent the induction of apoptosis (48). Therefore, PknE may prevent apoptosis of the infected macrophage by altering its cell wall to avoid triggering activation of TLRs associated with cell death.

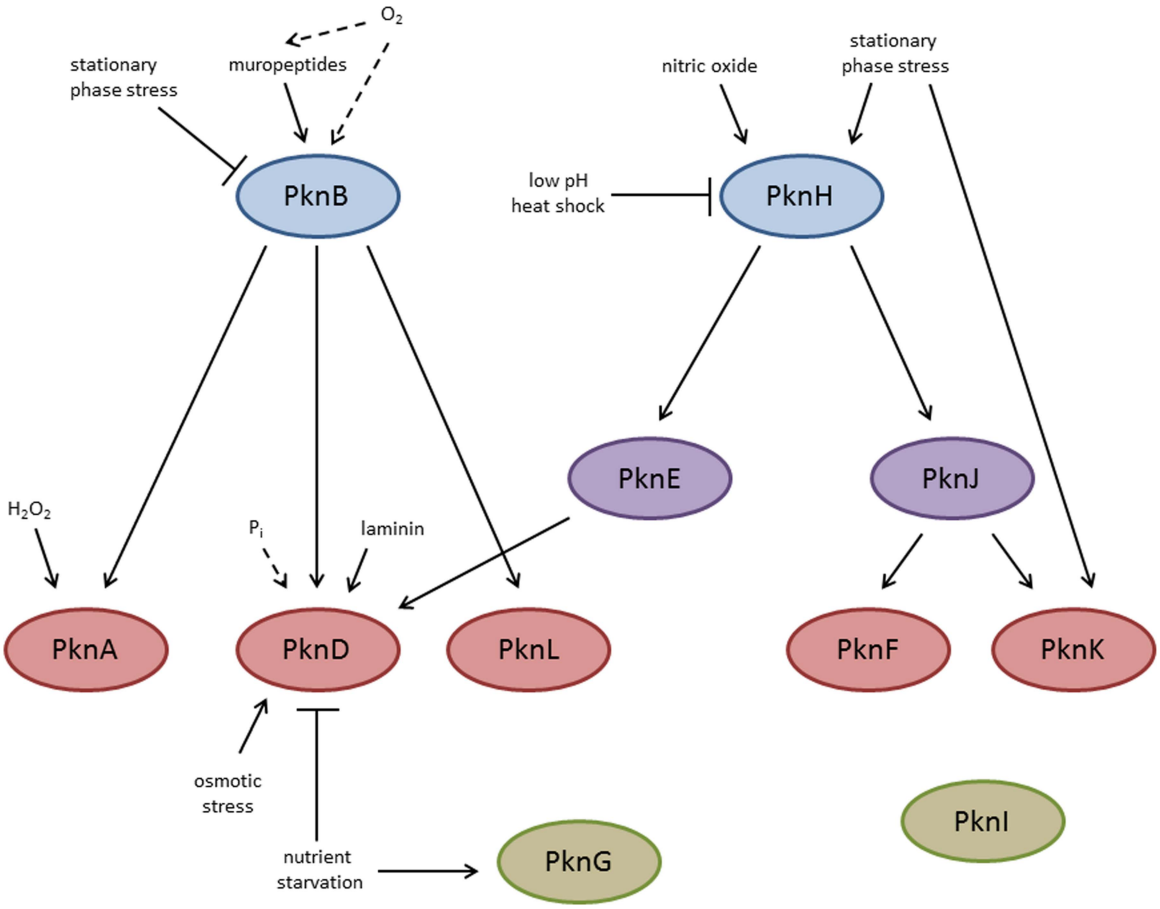


FIGURE 1 Hierarchy of *M. tuberculosis* STPK activation in response to extracellular and intracellular signals. Master STPKs (blue) sense environmental signals and further cross-phosphorylate the kinase domains of signal transducing (purple) and substrate (red) STPKs to propagate signals and regulate specific downstream proteins. (Figure modified from reference 28).

Another potential mechanism by which PknE may be involved in blocking apoptosis is through regulation of PtpA, which has been shown to be a substrate of PknE (51). Several studies have described substrates of PtpA in the host and its ability to directly interfere with host cell signaling pathways (16, 36–38). Dephosphorylation of one of the PtpA substrates, GSK3α, was indeed shown to decrease apoptosis of the host cell early in infection (36). Furthermore, a kinome analysis identified additional substrates for PtpA, including other apoptotic proteins (36). Therefore, it would be of interest to determine if PknE is able to suppress apoptosis through the regulation of PtpA.

Defense Against Host-Generated Reactive Oxygen and Nitrogen Species

Although *M. tuberculosis* is able to avoid immune recognition, the bacterium is still required to overcome the hostile environment of the phagosome. Upon phagocytosis, *M. tuberculosis* is immediately exposed to a considerable amount of reactive oxygen species, while reactive nitrogen species levels only increase after 72 h (52).

The alternate sigma factor, SigH, is a central regulator of mycobacterial adaptation to redox, heat, and acid stress and is induced upon phagocytosis of the macrophage (53). In addition to undergoing autoregulation of its promoter, SigH interacts posttranslationally with its cognate antisigma factor, RshA (54). Under oxidizing conditions the interaction between RshA and SigH is disrupted, leading to a strong induction of the SigH regulon. However, if the regulation of SigH was employed by this mechanism alone, by the time the intracellular environment became significantly oxidized and RshA dissociated from SigH, damage to *M. tuberculosis* biomolecules would have already occurred. Therefore,

TABLE 2 Growth and persistence phenotypes of *M. tuberculosis* serine/threoneine protein kinase mutants

STPK	*In vitro*[a]	Other *in vitro* conditions[b]	*Ex vivo* survival	*In vivo* survival
PknA[c]	Essential (29)			Negligible histopathology, no bacilli recovered from lung or spleen of mice (88)
PknB[c]	Essential (88)			Negligible histopathology, no bacilli recovered from lung or spleen of mice (88)
PknD	N.D.[f] (198)	↓ P_i poor conditions following 24 h starvation (199)	N.D. murine macrophages (173)	Required for invasion of brain endothelia (173) N.D. lungs for mouse or guinea pig (198)
PknE	N.D. (43)	↓ Dithiothreitol, glutathione, zinc, cadmium, ↑ Sodium nitroprusside, GSNO, acidified nitrite (43)	↓ 120 h THP-1 cells (43)	↑ Guinea pigs (200)[d] N.D. BALB/c mice (44)
PknF[e]	Faster growth, shorten cells (163)			
PknG	↓ Exponential, more pronounced stationary (41)	↓ Nutrient depleted (41)		↓ Lung, spleen, and liver in BALB/c and CD-1 mice (41)
PknH	N.D. (44)	↑ Acidified nitrite stress ↓ H_2O_2, O_2^- (44)	↓ 120 h THP-1 cells (44)	↑ Lung and spleen in BALB/c mice (44)
PknI	↓ Exponential (135)	↑ Acidic pH and hypoxia ↓ Acidic pH and oxygenated (135)	↑ 120 h THP-1 cells (135)	Hypervirulent SCID mice (135)
PknK	N.D. exponential ↑ Stationary phase Shortened cells (133)	↑ Acidic pH, hypoxia, H_2O_2 (133)	–	↓ Acute phase lung, ↑ persistent phase lung and spleen in C57BL/6 (133)

[a]*In vitro* growth under nutrient-rich, oxygenated conditions based on optical density or CFU data.
[b]*In vitro* growth conditions showing STPK mutant phenotypes varying from wild type. Based on optical density or CFU data.
[c]*In vivo* work performed with conditional depletion mutant of PknA or PknB.
[d]Polar effect was not ruled out due to lack of complemented strain.
[e]Results represent work performed with a *pknF* antisense mutant.
[f]N.D., no observed difference in phenotype.

the binding of RshA to SigH undergoes further kinase-mediated regulation by PknB, allowing *M. tuberculosis* to generate a more rapid response to an oxidative environment (55). To overcome challenge by oxidative stress, phosphorylation of RshA decreases its interaction with SigH *in vitro*, leading to increased SigH activity *in vivo*. In addition, phosphorylation of SigH was also demonstrated by PknB *in vitro*, but this modification had no effect on the SigH-RshA interaction. It is hypothesized that phosphorylation of SigH may alter binding or transcriptional activation at individual promoters under conditions of increased *pknB* expression; however, this remains to be determined.

In addition to PknB, PknA has also been shown to be involved in responding to oxidative stress. In the presence of H_2O_2, PknA increases its autophosphorylation levels (56). The implication of the observed enhancement of PknA activation was specifically observed to hinder proteasome assembly. The α-subunit (PrcA) and β-subunit (pre-PrcB) of the *M. tuberculosis* proteasome core complex are phosphorylated by both PknB and PknA, with PknB phosphorylation leading to enhanced degradation of the proteasomal substrate, Ino1 (56, 57). In contrast, phosphorylation of PrcA by PknA did not affect the proteasomal degradation *in vitro*. Rather, phosphorylation of both pre-PrcB and PrcA by H_2O_2-induced PknA activation inhibited the assembly of the holo-proteasome complex. Depletion of the proteasomal system was originally found to impair *M. tuberculosis* growth in the presence of NO but provided 2- to 3-fold greater resistance to H_2O_2 (58, 59). Thus, under conditions of oxidative stress, PknA inhibition of proteasome assembly is able to enhance *M. tuberculosis* resistance to H_2O_2 (56).

M. tuberculosis resistance to nitrosative stress has also been linked to the activity of STPKs. The production of NO in response to cytokines or pathogen-derived molecules is an important host defense mechanism against intracellular pathogens and has been shown to be essential in controlling *M. tuberculosis* infection (60, 61). Although *M. tuberculosis* is able to inhibit the colocalization of inducible nitric oxide synthase (iNOS) to the phagosomal membrane (62), it is unlikely that the bacilli can maintain this inhibition throughout the course of infection. Thus, it would be advantageous for the bacterium to possess additional protective mechanisms to ensure its survival. Deletion of either the *pknE* or *pknH* genes resulted in increased resistance to NO donors (43, 44, 46). However, the increased resistance to nitrosative stress of either mutant coincided with increased sensitivity to the oxidants tested. These findings implicate both PknE and PknH in sensing the host's redox environment and orchestrating a physiological response to enhance the survival of *M. tuberculosis*.

STPKs REGULATE *M. TUBERCULOSIS* MORPHOLOGY TO ENSURE COLONIZATION OF THE HOST

Bacterial proliferation can be thought of in two steps: elongation of the mother cell and division into two daughter cells. In the case of the tubercule bacillus, elongation is characterized by polar growth which requires the synthesis and incorporation of new materials into the cell wall. During the initial stages of infection, *M. tuberculosis* actively replicates inside the macrophage to ensure colonization of the host. The extensive regulation of the mycobacterial cell envelope biogenesis and division results in size and cell wall composition heterogeneity of daughter cells (63, 64). This physicochemical diversity is suggested to increase the survival odds for *M. tuberculosis* by enabling reservoirs within subpopulations that are able withstand diverse dynamic stressors encountered by the bacilli (65).

Cell Size

PknA and PknB have been shown to regulate the growth and morphology of the mycobacterial cell through a number of cell elongation and division proteins (29, 66). PknB is largely involved in regulating cell size through peptidoglycan biosynthesis (Table 1). PknB was shown to inhibit the acetyltransferase activity of GlmU, a protein involved in the synthesis of the peptidoglycan precursor UDP-*N*-acetylglucosamine (67). At the same time, the insertion of lipid II, the final intermediate in peptidoglycan biosynthesis, into the extracellular space is also regulated by PknB. In this situation, the membrane protein MviN responsible for the physical inversion of lipid II is negatively regulated by phosphorylation, ultimately impeding peptidoglycan biosynthesis (68). Furthermore, prior to insertion into the mycobacterial cell wall, lipid II undergoes extensive modification by a family of Mur synthases (69). These synthases are responsible for catalyzing the addition of acetyl, glycosyl, and amino groups to the peptide side chain of lipid II. Modification to peptidoglycan has been described to provide bacteria with resistance to hydrolysis by lysozymes, thereby limiting their detection by host pattern recognition receptors (70) as well as to regulate immunogenicity through the nucleotide-binding oligomerization domain-containing 2 immune receptor (71, 72). Both PknA and PknB have been shown to

interact with MurC-F, suggesting that they regulate the modification of muropeptides in response to the environment of *M. tuberculosis* (73, 74).

The final stages of peptidoglycan biosynthesis involve a family of penicillin binding proteins (PBPs) that are responsible for catalyzing cross-linking between peptidoglycan, a modification which also influences cell expansion. Traditionally, peptidoglycan precursors inserted into the cell wall are linked by transpeptidases to produce 4-3 cross-links. In contrast, up to 80% of *M. tuberculosis* peptidoglycan contains 3-3 peptide cross-links (75, 76), and this modification is crucial for persistence *in vivo* (77). Remodeling of peptidoglycan by the PBP DacB1 is believed to be responsible for maintaining 3-3 cross-links. Although the effect of phosphorylation on DacB1 activity is unknown, the PBP is a substrate of PknH *in vitro* (78). It is plausible that PknH regulates peptidoglycan cross-linking to increases during infection to promote cell wall rigidity and bacterial survival under stress. In addition, the PBP PonA1 is a substrate of PknB. Phosphorylation of PonA1 by PknB inhibits its transglycosylation activity and slows polar elongation, resulting in shorter cells (79). Lastly, PknA also coordinates peptidoglycan biosynthesis through the elongation complex, a macromolecular machine composed of peptidoglycan synthases and hydrolases that drive peptidoglycan remodeling during elongation. To localize elongation to the poles, Wag31 acts as an anchor at this site and provides a basis for the recruitment of the remaining complex components (80). Localization of Wag31 to cell poles is dependent on its phosphorylation by PknA, and growth of the *M. smegmatis* Wag31 phosphomimicking mutant resulted in shorter and wider cells (66).

In contrast to other bacterial species, mycobacterial septa are placed over a wide zone within the cell body (81, 82), further contributing to differences in daughter cell size as well as the distribution of proteins and small molecules between daughter cells (83). FtsZ, a homolog of eukaryotic tubulin, is the principal driving force of cytokinesis in mycobacteria. Through its self-activating GTPase activity, FtsZ undergoes polymerization to form a ring-like structure, known as the Z-ring (84), that was shown to be regulated by PknA among other mechanisms (85). This structure acts as a cytoskeletal scaffold for the recruitment and assembly of the divisome and provides energy for membrane constriction during cell division (86). Phosphorylated FtsZ showed a reduction in GTP hydrolysis and polymerization activity *in vitro*. Overexpression of PknA in *E. coli* resulted in phosphorylation of *E. coli* FtsZ and the production of elongated cells, indicating dysregulation in septum formation (85). In addition, the localization of PbpA, a peptidoglycan synthase part of the divisome, to the septum is thought to be mediated via phosphorylation by PknB (87). PbpA is a substrate of PknB *in vitro*, and the absence of phosphorylated PbpA prevents PbpA localization to the septa and causes the elongated growth of *M. smegmatis in vitro* (87).

As outlined earlier, PknA and PknB play a crucial role in polar elongation and septal localization. Therefore, these two kinases are likely responsible, in part, for differences in the size of daughter cells observed *in vivo*. This notion is supported by the fact that overexpression of these kinases results in short bulging cells, while their depletion causes narrow and elongated bacilli (66). Not only are PknA and PknB essential for *M. tuberculosis* growth *in vitro*, but they also are indispensible in the survival and pathogenesis of *M. tuberculosis* during murine infection (29, 88) (Table 2). Thus, PknB senses host environmental factors that enable its orchestration of downstream signaling to tightly regulate growth and generate a heterogeneous population that enables *M. tuberculosis* to persist in the presence of host innate and acquired immunity.

Cell Wall Composition

Transcriptional analysis of *M. tuberculosis* isolated from tuberculosis patients identified substantial changes in the expression of cell wall biosynthetic genes, including the upregulation of lipid synthesis genes (89). Thickening of the cell wall restricts the transit of toxic molecules including antibiotics. Lipids can also act as a sink for toxic by-products generated by β-oxidation during *in vivo* growth (90), absorb oxidative radicals (91), and manipulate the host immune response (92). Of these lipids, mycolic acids, phthiocerol dimycocerosate (PDIM), and sulfolipid 1 (SL-1) were all found to increase in abundance and/or alter their composition during *M. tuberculosis* growth *in vivo* (89, 93, 94).

The outer layer of the *M. tuberculosis* cell wall is composed of long-carbon-chain mycolic acids that give rise to the observed thick waxy coat and the remarkable impermeability of mycobacteria (95). The biosynthetic pathway of mycolic acids begins with the *de novo* synthesis of fatty acids from acetyl-CoA. The mycobacterial fatty acid synthase (FAS) II is composed of four sets of enzymes that are essential in catalyzing each cycle of elongation: β-ketoacyl-ACP reductase (MabA), β-hydroxylacyl-ACP dehydratases (HadAB/HadBC), NADH-dependent *trans*-2-enoyl-ACP reductase (InhA), and β-ketoacyl-ACP synthases (KasA or KasB). Inter-

estingly, each FAS-II enzyme is phosphorylated by multiple STPKs (Table 1), enabling regulation of mycolic acid biosynthesis in response to variable growth environments (96–101). STPKs are also involved in reducing the production of the FAS-II system precursors by phosphorylating malonyl-CoA-ACP (FabD) (101) and the β-ketoacyl-ACP synthase (FabH) (102). Being the target of multiple STPKs and the fact that phosphorylation of individual enzymes results in only the partial reduction of its activity enables fine-tuning of the FAS-II system (Table 3). Furthermore, the observation that PknA and PknB negatively regulate these enzymes indicates that even under conditions of growth, mycolic acid biosynthesis is being constrained. Mycolic acid biosynthesis is an expensive process, and in addition, *M. tuberculosis* resides in nutrient-limited phagosomes. Thus, it would be beneficial to carefully balance mycolic acid biosynthesis with cell expansion.

Differentially regulating FAS-II enzymes may also provide opportunity for the full extension of mycolic acids. The importance of extending these chains has been observed in the *M. tuberculosis kasB* mutant. Production of shorter mycolates in this strain resulted in impaired growth, increased cell wall permeability, and severe defects in resisting host defenses and antibiotic action (103, 104). Lastly, it has also been proposed that mycolic acids are recycled under conditions that damage the cell envelope (105, 106). Consistent with this notion, gene expression profiling of *M. bovis* BCG suggests that "new" mycolic acids are synthesized via the remodeling of older chains during infection (107).

The *mymA* operon (*rv3803-rv3809*) is predicted to encode for the gene products involved in an alternative approach for the condensation of long fatty acids for the synthesis of mycolic acids (108). *M. tuberculosis* disrupted in the *mymA* genes has impaired survival in both activated macrophages and guinea pigs as well as increased cell wall permeability (109). The *mymA* operon has considerable basal activity, which is further enhanced 2- to 3-fold by the transcriptional regulator, VirS, under acidic conditions (14, 109, 110). Interestingly, VirS undergoes posttranslational modification by PknK, enhancing its DNA binding affinity for the *mymA* promoter; however, PknK was only found to stimulate VirS-mediated transcription of the *mym* promoter under physiological conditions (14). Therefore, in the absence of acid stress, PknK may enhance VirS activity, modulating mycolic acid biosynthesis through a FAS-II-independent pathway during infection.

In the past decade, evidence of STPK involvement in PDIM biosynthesis and export has slowly been accumulating. PDIM is implicated in protecting *M. tuberculosis* from reactive nitrogen species generated by the host (111), and thus it is not surprising that PknH, activated by nitrate stress (44, 112), has been found to positively regulate PDIM biosynthesis (113). The exact mechanism behind PknH regulation of PDIM biosynthesis is unknown, but it could be speculated that nitrate stress leads to the downstream activation of PknD and PknE, which have targets in PDIM transport (114) and biosynthesis (115), respectively. In addition to PknE, PknB was also found to phosphorylate PapA5, an acyltransferase that catalyzes the dual esterification of mycocerosate onto phthiocerol to complete the biosynthesis of PDIM (115, 116). Although identified as substrates of *M. tuberculosis* STPKs, it remains unknown what effect phosphorylation has on the activity of PapA5 or the PDIM transporter, MmpL7. Since PknE is also implicated in sensing nitrate stress (43), it is suspected that phosphorylation of PapA5 may positively regulate its enzymatic activity.

PDIM and sulfolipid-1 (SL-1) production are coupled via the metabolic flux of MMCoA (93), and as a result STPK downregulation of PDIM biosynthesis would lead to an increase in SL-1 production. SL-1 has been found to negatively regulate *M. tuberculosis* growth in human macrophages as well as provide protection against human cationic antimicrobial peptides *in vitro* (117). Enhancing PDIM production via STPK would reduce the quantity of SL-1 (93) while increasing *M. tuberculosis* resistance to reactive nitrogen species (111). The early immune response to *M. tuberculosis* is also subdued by the presence of PDIM, which is shown to inhibit the secretion of tumor necrosis factor-α and interleukin-6 from resting macrophages and dendritic cells (111). Therefore, STPK regulation of PDIM production and export may contribute to the intracellular growth and survival of *M. tuberculosis* during the initial stage of infection.

STPKs COORDINATE *M. TUBERCULOSIS* PHYSIOLOGY TO ACHIEVE NONREPLICATING PERSISTENCE

As the disease progresses, *M. tuberculosis* can further diversify in response to pressure from anatomical location, the host immune response, and drug treatment. These adaptations lead to differences in gene expression profiles, metabolism, growth rate, and other functional characteristics, resulting in a heterogeneous population of bacteria (118). Often, conditions in the host give rise to a subpopulation of dormant-like bacteria characterized

TABLE 3 Effect of phosphorylation on *M. tuberculosis* serine/threonine protein kinase substrates[a]

Substrate	Function	Effect of phosphorylation	Kinase	References[b]
DosR	Dormancy	Enhances its binding activity to dosR regulon promoter	PknH	27
EF-Tu	Cell division	Reduces interaction with GTP	PknB	190
EgtD	EGT biosynthesis	Inhibits methylation activity	PknD	132
EmbR	Arabinan biosynthesis	Enhances EmbR binding to *embCAB* promoter	PknH	25
FabD	MA biosynthesis	Decreases its condensing activity	PknF	96
FipA	Cell division	Enhances interaction with FtsZ	PknA	179
FtsZ	Cell division	Impairs GTP hydrolysis and polymerization	PknA	85
GarA	TCA cycle	Inhibits binding to KDH, GDH, and GltS	PknB	40, 167
			PknG	40
GlgE	α-glucan biosynthesis	Decreases maltosyltransferase activity	PknB	143
GlmU	PG biosynthesis	Decreases acetyltransferase activity	PknB	67
HupB	Cell division	Inhibits its DNA binding activity	PknE	185
InhA	MA biosynthesis	Decreases enoyl reductase activity	PknA	98
			PknB	98
			PknE	98
			PknL	98
KasB	MA biosynthesis	Decreases its condensing activity	PknF	100
L13	Oxidative stress	Promotes its association with RenU and enhances RenU hydrolysis of NADH	PknG	193
MabA	MA biosynthesis	Decreases β-ketoacyl-ACP reductase activity	PknB	96
Mdh	TCA cycle	Inhibits dehydrogenase activity	PknD	172
MviN	PG biosynthesis	Induces dimerization with FhaA	PknB	68
OprA	Osmotic stress	Enables SigF binding to RNA polymerase	PknD	31
ParB	Cell division	Inhibits DNA binding to *parS* and interaction with ParA	PknA	180
			PknB	180
			PknD	180
			PknF	180
PonA1	PG biosynthesis	Inhibits transglycosylation activity	PknB	79
PrcA	Proteasome	Inhibits proteasome assembly	PknA	56
		Enhances degradation of Ino1	PknB	56
PstP	Signaling	Decreases phosphatase activity	PknA	183
PtpA	Signaling	Enhances phosphatase activity	PknA	51
RshA	Stress response	Inhibits its interaction with SigH	PknB	55
Rv0516c	Anti-antisigma factor?	Inhibits association with Rv268	PknD	30
Rv1747	ABC transporter	Enhances enzymatic activity	PknF	201
Rv2175c	DNA binding?	Inhibits its DNA binding	PknL	26, 197
SahH	Methionine cycle	Decreases hydrolase activity	PknA	182
		Decreases hydrolase activity and its affinity to NAD^+	PknB	182, 189
			PknD	182
			PknE	182
			PknF	182
			PknL	182
VirS	MA biosynthesis?	Enhances binding to *mym* promoter under physiological conditions	PknK	14
Wag31	Cell division	Localizes it to the cell poles and enhances oligomerization of the elongation complex	PknA	202

[a]Abbreviations: EGT, ergothioneine; GDH, glutamate dehydrogenase; KDH, α-ketoglutarate dehydrogenase complex; MA, mycolic acid; TCA, tricarboxylic acid; PG, peptidoglycan.

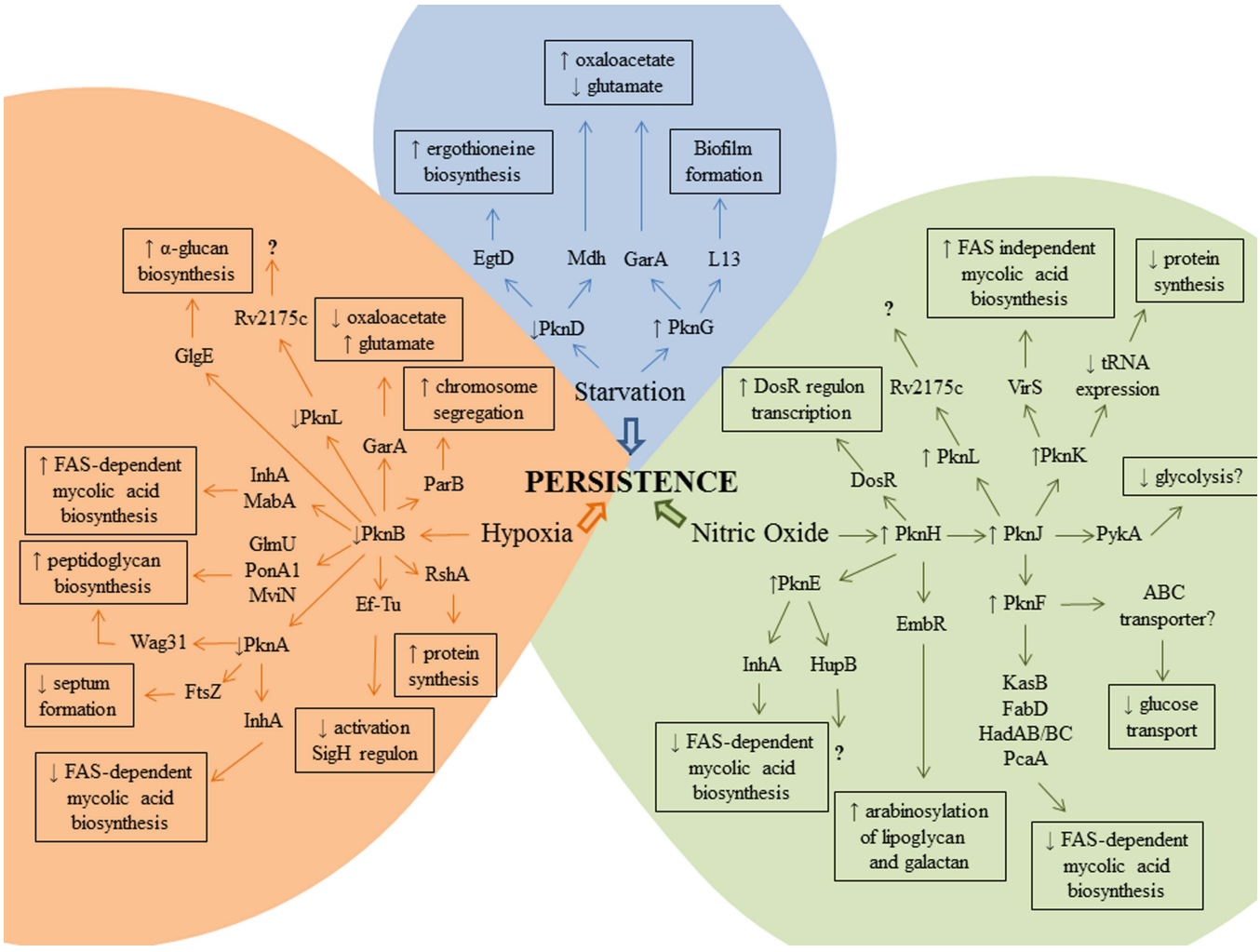
[b]Reference of the effect of phosphorylation on the target substrate identified *in vitro* and/or *in vivo*. Only cases where the effect of phosphorylation by the specific STPK tested and/or phosphorylation sites are mentioned in the table. We do not assume that the STPKs phosphorylate their substrates on the same residues and have the same effect. Table only includes analysis of *M. tuberculosis* proteins.

as nonreplicating with low metabolic activity. It is these dormant-like bacteria that are the reservoir for *M. tuberculosis* persistence and reactivation of the disease. To successfully enter a dormant-like state, *M. tuberculosis* senses a number of unfavorable growth conditions resulting in growth arrest, cell wall remodeling, and downregulation of metabolism (Fig. 2).

Growth Arrest

M. tuberculosis infection is primarily characterized by the formation of granulomas, organized structures of immune cells that act to control and prevent the dissemination of infection. Despite the heightened immune response that is usually associated with granulomas, *M. tuberculosis* is still able to persist long-term within this structure. Survival under these conditions is likely due to arrested growth and cellular respiration of *M. tuberculosis* in response to NO generated from activated macrophages and the hypoxic environment of the granuloma (119, 120). The *M. tuberculosis* state of quiescence, termed nonreplicating persistence (NRP), is entered upon the activation of the DosR regulon, a set of 48 genes that downregulate cellular respiration. DosR is regulated by two cognate sensor kinases, DosS and DosT (121, 122), which activate the DosR regulon under conditions of hypoxia (123–125), NO (120), and carbon monoxide (126, 127). Interestingly, PknH was shown to sense host NO and trigger the induction of the DosR regulon (27, 44, 112, 128). Further evidence describes *pknH* transcription to be upregulated during

FIGURE 2 *M. tuberculosis* STPK cell signaling network associated with persistence. STPKs sense specific environmental cues (starvation, hypoxia, and nitric oxide) and coordinate a physiological response that triggers *M. tuberculosis* to enter a state of nonreplicating persistence.



stationary phase growth *in vitro* (129), and the *pknH* deletion mutant showed higher bacillary loads in mouse organs than did wild type (44). Taken together, these findings implicate PknH in slowing the growth of *M. tuberculosis* to achieve a state of NRP during infection, and perhaps specifically in response to the granuloma's environment (Fig. 2).

The induction of NRP is not limited to the redox environment of *M. tuberculosis*. The other well-known contributing factor leading to NRP is nutrient deprivation (Fig. 2). Both the phagosome and granuloma are sites of nutrient deprivation for pathogens, and *M. tuberculosis* isolated from lung lesions demonstrated an altered morphology and staining properties that were similar to cultures starved in distilled water for two years (130). Furthermore, Loebel et al. (131) and Betts et al. (128) identified that starvation of *M. tuberculosis* in phosphate-buffered saline resulted in the gradual shutdown of respiration to minimal levels, and the bacteria remained viable but nonreplicating. Among the 323 genes involved in adaptation in this model of persistence, *pknB* and *pknD* were both found to be significantly downregulated (128). As a result, it is tempting to speculate that PknB senses the extracellular signal of starvation terminating downstream STPK signaling pathways involved in cell proliferation via posttranslational modification. However, regulation of bacteriostasis by PknB was shown to be specific to oxygen levels, rather than nutrient starvation (33).

PknD also has a functional extracellular domain (32), and its kinase activity is regulated under a variety of processes (Fig. 1), suggesting broad use of this kinase by *M. tuberculosis*. However, the relevance of the downregulation of PknD during nutrient deprivation (128) became apparent when we discovered that PknD negatively regulates ergothioneine biosynthesis in *M. tuberculosis* (132). Ergothioneine is a sulfur containing amino acid derived from histidine, and very little is known about its physiological role in microorganisms. However, ergothioneine is required for *M. tuberculosis* survival under long-term starvation (132), suggesting that the downregulation of PknD is essential for persistence. In contrast, nutrient starvation (128) and hypoxia (33) show no influence on the transcript levels and activity of PknH *in vitro*.

PknK and PknI have also been implicated in slowing the growth of mycobacteria *in vitro*, which was further shown to be relevant because the *M. tuberculosis* Δ*pknK* and Δ*pknI* mutants both demonstrate enhanced growth during macrophage and/or mouse infection (133–135). PknH indirectly regulates the activity of PknK through the signal transduction kinase PknJ (28) (Fig. 1); however, PknK is also positively regulated at a transcriptional level during stationary phase (133). The slowing of *M. tuberculosis* growth is thought to be the consequence of PknK regulating the expression of a variety of genes, including those involved in cell wall processes and lipid metabolism. Perhaps the most notable observation from this study was the inhibitory effect on transcription and translation processes of tRNAs resulting in the repression of protein synthesis in *M.* tuberculosis (134). The exact mechanism of how PknK regulates the expression of a variety of genes is unknown; however, it is plausible that PknK targets a variety of transcriptional regulatory proteins, as observed in the case of VirS (14), to ultimately slow *M. tuberculosis* growth.

PknI is not part of the *M. tuberculosis* STPK interaction network described by Baer et al. (28), and little is known about its activation (Fig. 1). The Δ*pknI* mutant showed enhanced growth under acidic pH and limited oxygen availability (Table 2), suggesting that PknI is involved in slowing *M. tuberculosis* growth in response to the macrophage environment (135). However, two independent groups have shown that *pknI* expression is not induced upon infection of the macrophage (133, 136), again suggesting that expression data must be interpreted with caution for STPKs. However, it remains possible that PknI expression is not detected during infection because the signaling cascade is only induced at very early stages of infection (<18 h), an event similar to what is observed with PknK (133). Alternatively, the initial expression levels of PknI may be sufficient for its activity within macrophages (135). Currently, there is a single known *in vitro* substrate for PknI: FabD (Table 1); however, the effect of phosphorylation remains to be investigated. In addition, due to its positioning in the *M. tuberculosis* genome, PknI has been proposed to play a role in cell wall synthesis and division (12).

Cell Wall Remodeling

M. tuberculosis halts cell division and undergoes extensive cell wall remodeling while transitioning into a state of NRP (137). Cunningham and Spreadbury (138) reported a very prominent thickening of the cell wall outer layer in *M. tuberculosis*, which was later attributed to the gradual accumulation of loosely bound extracellular material around the bacilli in the form of a capsule (129). This matrix is primarily composed of proteins and polysaccharides (139), with only a small proportion (2 to 3%) containing lipids (140). The major carbohydrates making up 80% of the extracellular capsule are α-glucans, and *M. tuberculosis* strains defective in

the production of capsular α-glucans showed attenuated survival in mice during the persistence phase of infection (141).

The biosynthesis of α-glucan in *M. tuberculosis* occurs through three known pathways. Of these, the GlgE pathway has been described as a nonclassical type responsible for the conversion of trehalose into branched α-glucan through maltose 1-phosphate (142). In this pathway the linear backbone of α-glucans is directly synthesized from maltose 1-phosphate by the essential maltosyltransferase, GlgE. To ensure the appropriate channeling of trehalose in the formation of the cell wall, it is expected that the GlgE pathway would be negatively regulated during cell growth. Consistent with this idea, Molle and colleagues (143) identified PknB to negatively regulate GlgE activity and ultimately α-glucan biosynthesis.

Another mechanism employed by *M. tuberculosis* to adapt to a state of NRP is to increase the abundance of free mycolates in the cell wall lipids (129). Free mycolates have been shown to play a key role in the formation of mycobacterial pellicle biofilms (144), and *M. tuberculosis* biofilms have been observed within the caseum of human granulomas (119). The production of free mycolates occurs as the result of the direct cleavage of trehalose dimycolate (145), and free trehalose is then transported back to the cytoplasm (146). Since *pknB* transcripts are downregulated in the absence of *M. tuberculosis* growth (66, 128), it is plausible that α-glucan biosynthesis via GlgE would thereby increase, contributing to the observed thickening of the *M. tuberculosis* capsule during NRP.

Remodeling of the mycobacterial cell wall during NRP was also found to include increased arabinosylation and abundance of lipoglycans in response to *in vitro* nutrient starvation (129). In mycobacteria, the lipoglycans, lipomannan and lipoarabinomannan (LAM), are associated with the cell wall and are exposed on the outer surface of the bacterium (147). Both of these lipoglycans are ligands of the TLR2; however, lipomannan is a stronger inducer of this receptor's response relative to LAM (148). EmbC is an arabinofuranosyltransferase which serves to elongate the arabinan domain of LAM (149). Transcriptome analyses identified *embC* to be upregulated during stationary phase (129, 149), which most likely contributes to the corresponding increase in LAM arabinosylation observed during NRP (129). The enhanced expression of *embC* is indirectly regulated by PknH through the phosphorylation of the response regulator EmbR (25). Additionally, *pknH* is also upregulated during stationary phase (129). These results implicate PknH in sensing a growth-limiting factor, resulting in a signaling cascade to increase *embC* expression and ultimately the arabinose content of LAM.

In vitro environmental conditions, such as anoxic and nutrient starvation, responsible for directing *M. tuberculosis* into a state of NRP are also found to produce cultures that lose their acid-fastness (130, 150–152). The involvement of mycolic acids in acid-fast staining arose when Jacobs and colleagues (103) observed that the deletion of *kasB* resulted in a loss of acid-fastness. Furthermore, this *M. tuberculosis kasB* mutant displayed significant *in vivo* growth attenuation that led to a long-term persistent infection reminiscent of latent tuberculosis. Prior to these findings, the condensing activities of KasB were found to be under the regulation of STPKs (101), implicating these kinases as major players in the progression of the disease to a latent state. Interestingly, the original study identifying KasB as a substrate of multiple STPKs identified PknA to positively regulate KasB; however, more recent work by the same group showed that phosphorylation of KasB by PknF resulted in shortened mycolic acids that lacked *trans*-cyclopropanation (100). The relevance of the *in vitro* observation of KasB phosphorylation by PknA remains questionable. Nonetheless, further analysis of the KasB phosphomimetic mutant showed a complete loss of acid-fast staining as well as the incapacity to grow yet establish a long-term persistent infection in mice (100).

The core structure of mycolic acids is conserved across mycobacteria; however, pathogenic mycobacteria produce significant quantities of cyclopropanated mycolic acids. Following elongation, the meromycolic chain produced by the FAS-II system can undergo cyclopropanation by a number of *M. tuberculosis* methyltransferases (MmaA 1 to 4, PcaA, and CmaA2). PcaA and MmaA2 have been analyzed for their ability to act as substrates for *M. tuberculosis* STPKs, with only PcaA acting as a substrate for PknF and PknH (153). PcaA methyltransferase activity was decreased upon phosphorylation by PknF. Since *M. tuberculosis* PcaA is implicated in persistence (154) and attenuated immunopathology (155), these findings suggest that mycolic acid modifications have an immunomodulatory function, and specifically, cyclopropanation acts to suppress the immune response to *M. tuberculosis*. However, in the presence of NO, PknH and its downstream signaling effectors, including PknF, are activated, leading to the negative regulation of mycolic acid elongation and cyclopropanation (27, 28). This regulatory mechanism may function to simply act as part of the observed

shutdown of mycolic acid biosynthesis when *M. tuberculosis* enters a dormant state (128, 156) and/or may be used to modulate the immune response later in infection (157, 158).

Slowing Central Metabolism

Based on early observations by Bloch and Segal (159), it is accepted that *M. tuberculosis* preferentially utilizes fatty acids as a carbon source during infection. Although the *M. tuberculosis* genome encodes for transporters and enzymes known to metabolize sugars (160), the conditions in which carbohydrates can be utilized for *in vivo* growth still need to be determined. Interestingly, a double mutant of *M. tuberculosis* glucokinases, the enzymes responsible for generating glucose-6-P in the first step of glycolysis, was unable to persist during the chronic phase of infection (161). From these findings, it remains plausible that *M. tuberculosis* has access to glucose during infection; however, the production of glucose-6-P may, rather, be the consequence of gluconeogenesis. The regulatory mechanisms of glucose metabolism further support the notion that glucose availability is limited inside the host. The activity of the glycolytic enzyme pyruvate kinase A (PykA) has been shown to be a substrate of PknJ in *M. tuberculosis* (162). Although the effect of phosphorylation was not studied in depth, a phosphomutant of PykA suggests negative regulation. As PknJ is activated by PknH, decreasing PykA activity during NRP would impede the production of pyruvate, enabling gluconeogenesis to proceed. Furthermore, glucose transport has also been shown to be mediated by STPKs. *M. tuberculosis* expressing antisense *pknF* demonstrated an increase in [^{14}C] glucose uptake *in vitro* (163). Molle et al. (164) identified Rv1747 to be a substrate of PknF, suggesting that other ABC transporters could be regulated by this mechanism and mediate glucose transport. PknJ (165) and PknF (163) have been shown to decrease the growth rate of *M. bovis* BCG and *M. tuberculosis*, respectively. Perhaps, together PknJ and PknF coordinate the shutdown of carbohydrate catabolism during the *M. tuberculosis* life cycle in the host to slow bacterial growth and prepare the cell for entering a quiescent state.

The observed shutdown of the glycolytic pathway during infection and the inability of carbohydrate transporters to promote the survival of *M. tuberculosis in vivo* suggest that glucose is primarily generated via gluconeogenesis (161). This thought is consistent with the dramatic loss of virulence in a mutant strain that lacks the gluconeogenic enzyme, phosphoenolpyruvate kinase (166). During stationary phase and when grown in nutrient-depleted media, an *M. tuberculosis* mutant deficient in PknG displays reduced growth, and upon further analysis was found to accumulate both glutamate and glutamine (41). Glutamate is one of the major gluconeogenic precursors for cells. The amino acid is broken down by glutamate dehydrogenase to produce α-ketoglutarate and ammonium. A small forkhead associated domain protein known as GarA regulates α-ketoglutarate entry into the tricarboxylic acid (TCA) cycle. GarA binds and inhibits both the α-ketoglutarate dehydrogenase complex and glutamate dehydrogenase (40). In the meantime, GarA also promotes glutamate synthesis by activating glutamine oxoglutarate aminotransferase, an enzyme which assimilates glutamine together with α-ketoglutarate to produce glutamate (167). PknG was shown to phosphorylate GarA and prevent binding to its enzyme partners. Therefore, GarA phosphorylation impedes glutamate synthesis and relieves TCA cycle inhibition in *M. tuberculosis* (40).

PknG has been shown to be upregulated during infection (133) and contribute to the survival of a number of mycobacterial species both in macrophages (39, 168–170) and in mice (41, 168). The macrophage phagosome is presumed to be nutrient-poor, which likely results in the activation of PknG-mediated signaling to upregulate gluconeogenic pathways (Fig. 2). It should be considered that during *in vivo* growth, gluconeogenesis may be acting to support the anabolism of cell wall components, such as mannosylated LAM (171), or other pathways, which in turn results in the blockage of phagosome-lysosome fusion, a phenotype noted earlier to be associated with PknG. A second enzyme involved in gluconeogenesis and the TCA cycle, malate dehydrogenase, is also a substrate of a number of STPKs, including PknD (172). Phosphorylation by PknD has a negative effect on malate dehydrogenase activity. Thus, in conditions resulting in nutrient starvation where *pknD* transcripts are reduced, one would expect an increase in oxaloacetate. In addition, PknD is not required for growth in macrophages (173), indicating that this kinase may also contribute to the regulation of gluconeogenesis during infection.

THE SWITCH: SENSING WHEN TO EXIT NRP

Little is known about the environmental conditions that are associated with the transition between latency and reactivation. Previous work has shown that growth of hypoxia-arrested *M. tuberculosis* occurs upon reaeration of *in vitro* cultures (33, 174). These findings are consistent with anatomically related reactivation,

which commonly occurs in the upper lobes, the area of the lungs that have the highest oxygen tension (175), while bacterial dormancy is associated with the oxygen-limited granuloma (176, 177). The regulation of oxygen-dependent replication is mediated in part by PknB (33). PknB protein levels were found to be upregulated in response to oxygen, where PknB decreased during hypoxia. Regulation of bacteriostasis was found to be limited to oxygen, because other conditions known to inhibit growth such as nitric oxide, low pH, and nutrient starvation had no effect on the growth and survival of a *pknB* overexpression strain (33).

The exact mechanism by which PknB senses changes in oxygen tension still needs to be defined, because its extracellular PASTA domains are not believed to be involved in oxygen sensing (178). It is thought that *M. tuberculosis* uses resuscitation-promoting factors (Rpfs) to initiate regrowth following dormancy (118). *Micrococcus luteus* Rpf orthologs possess a conserved domain predicted to have lysozyme activity and may therefore cleave peptidoglycan and in turn activate PknB during reaeration. Alternatively, PknB activation was suggested to go through cross-phosphorylation by another STPK or two-component signaling system in response to oxygen levels (33) as previously exemplified (27).

From the above findings, it is clear that PknB is critical in transducing growth and replication signals in response to oxygen levels. Careful regulation of its activity is required at every stage of the life cycle of *M. tuberculosis*. As suggested, PknB may therefore represent a highly vulnerable drug target for *M. tuberculosis* during both active and latent disease.

CONCLUSION

To establish persistence, *M. tuberculosis* STPKs regulate mycobacterial proteins to adapt the bacilli's physiology. Furthermore, to ensure survival inside the phagosome, *M. tuberculosis* interferes with the host intracellular signaling of the infected macrophage through the secreted phosphatases PtpA and SapM. Currently, little evidence exists that demonstrates secretion and direct association of *M. tuberculosis* STPKs with host effector proteins. Rather, the role STPKs play in evading the host immune response is mainly through the regulation of cell growth, cell wall remodeling, and the activation of specific stress responses during infection.

Generally, as shown in Table 1, multiple STPKs can act on a defined single substrate and have a similar effect, suggesting that *M. tuberculosis* carefully fine-tunes its physiological response to match the associated conditions of the bacterium. In addition, multiple enzymes that belong to the same pathway or cell process are also regulated by multiple STPKs. Such regulatory flexibility can lead to a population differing in cell size, growth rate, and cell wall composition. As a result, the heterogeneity of this population may improve the fitness of *M. tuberculosis* by providing bacteria with the physiological diversity to successfully grow in a range of host microenvironments or persist in a quiescent state.

Currently, our knowledge of the conditions that activate STPKs during infection is limited. Therefore, it is difficult to link the physiological adaptation of *M. tuberculosis* with the host response. We can predict that *M. tuberculosis* replicates during the initial stage of infection as well as during disease reactivation, a process which is regulated for the most part by PknA and PknB. However, under conditions of limited oxygen and nutrients as well as in the presence of NO, *M. tuberculosis* enters a state of NRP to ensure its long-term survival in the host. Although these conditions can be encountered at any point during the life cycle of the bacilli, *M. tuberculosis* primarily exists in a state of NRP in the granuloma. As summarized in Fig. 2, the hypoxic environment of the granuloma likely shuts down PknB and its downstream signaling pathway to arrest growth and increase peptidoglycan and α-glucan biosynthesis to enhance rigidity and thickness, respectively, of the cell wall. In parallel, *de novo* biosynthesis of mycolic acids is downregulated by both the activation of PknH and the inhibition of PknB signaling cascades to potentially reduce *M. tuberculosis* immunogenicity. The activation of PknH by NO generated from activated macrophages additionally enhances the induction of the DosR regulon to further prevent bacterial replication and inhibit aerobic respiration. In addition to DosR, PknH coordinates slowing of *M. tuberculosis* growth through PknJ and its downstream targets PknF and PknK to inhibit glucose catabolism and protein synthesis. Despite reduced metabolic activity during NRP, *M. tuberculosis* relies on gluconeogenesis for biomass production and survival throughout infection. It is likely that PknG plays a crucial role in this process through its regulation of GarA. Furthermore, the downregulation of PknD is potentially crucial for the survival of *M. tuberculosis* during NRP due to its role in gluconeogenesis and ergothioneine biosynthesis.

Understanding the mechanisms behind host signaling pathways targeted by *M. tuberculosis* as well as how the bacilli physiologically adapts to persist within the host are crucial for effective management of chronic

infection. In the past two decades, the field of oncology has been dedicated to developing libraries of compounds that block protein kinase activity due to their role in uncontrolled cell division. Therefore, it may be possible to use our knowledge of how *M. tuberculosis* interferes with host signaling pathways and enhance the immune response with an appropriate compound. Alternatively, screening these libraries for antimicrobial activity against *M. tuberculosis* may also prove beneficial. Because *M. tuberculosis* STPKs play a crucial role in regulating a wide variety of cell processes involved in bacterial growth and persistence, altering the activity of these kinases may represent a promising approach for novel drug discovery.

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Author Queries

Q1: Is tyrosine correct for "Tyr"?
Q2: Shouldn't "11 STPKs" be "12 STPKs" if they're A to L?
Q3: "In contrast His-kinases…" Should this be "In contrast to His-kinases…"? If not, something is missing/wrong elsewhere in the sentence.
Q4: Please spell out MMCoA.
Q5: "…reactivation, which commonly occurs…" Okay as edited?
Q6: Please spell out or explain PASTA.
Q7: Ref. 119: Please add editor(s) names.
Q8: Table 1: Please spell out MA and PG. (biosynthesis)
Q9: Table 2: Per house style, dashes are not used to indicate "no information" in tables. If the dashes mean something else, that can be defined in the table footnotes.
Q10: Table 2: Is sodium nitroprusside correct for SNP? Please spell out GSNO.
Q11: Table 2, PknF row: Is "shorten" correct? Not "shorter"?
Q12: Table 2, PknH row: Please spell out THP.
Q13: Table 2, footnote: Is optical density correct for OD?
Q14: Table 3, L13 row: Please define RenU.