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Metabolic Perspectives on Persistence

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SUMMARY

Accumulating evidence has left little doubt about the importance of persistence or metabolism in the biology and chemotherapy of tuberculosis. However, knowledge of the intersection between these two factors has begun to emerge only recently. Here, we provide a focused review of metabolic characteristics associated with *M. tuberculosis* persistence. We focus on metabolism because it is the biochemical foundation of all physiologic processes and a distinguishing hallmark of *M. tuberculosis*'s physiology and pathogenicity. In addition, it serves as the chemical interface between host and pathogen. However, existing knowledge derives largely from physiologic contexts in which replication is the primary biochemical objective. The goal of this review is to reframe existing knowledge of *M. tuberculosis* metabolism in the context of persistence where quiescence is often a key distinguishing characteristic. Such a perspective may help guide ongoing efforts to develop more efficient cures and inform on novel strategies to break the cycle of transmission sustaining the pandemic.

INTRODUCTION

DNA evidence indicates that *Mycobacterium tuberculosis* (*M. tuberculosis*) and humans have co-habited one another since the emergence of *Homo sapiens* as a species (1). Within humans, *M. tuberculosis* has been found to reside chiefly within and amidst cells of the immune system. *M. tuberculosis* has thus evolved in close physical and functional proximity to host immunity.

In most hosts, *M. tuberculosis* occupies the majority of its natural life cycle in a clinically asymptomatic state of slowed or arrested replication. However, *M. tuberculosis* is widely recognized for its ability to cause clinical disease in immunocompetent hosts years, if not decades, after successful containment of primary infection, and successfully transmit itself to new hosts. This suggests that *M. tuberculosis* has evolved specific mechanisms to sense, withstand and recover from prolonged periods of immune-imposed suppression; a trait that became evident with the discovery of increased rates of disease and mortality in immunosuppressed and/or -deficient populations (2).

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In addition to host immunity, *M. tuberculosis* is equally well recognized for its ability to persist in the face of even effective, chemotherapy, a trait often referred to as non-heritable antibiotic resistance or phenotypic tolerance. Phenotypic tolerance is widely believed to explain the need for treatment durations longer than for virtually any other bacterial infection which, in turn, have inadvertently promoted the emergence of drug resistance itself (3).

Persistence has thus emerged as a central feature of both *M. tuberculosis*'s physiology and pathogenicity. However, like most phenotypic traits, persistence has come to encompass an increasingly diverse and heterogeneous array of physiologic states and mechanisms. For *M. tuberculosis*, persistence has been linked to both deterministically- and stochastically-encoded programs in majority and minority subpopulations, respectively (4). The number and nature of states and/or mechanisms mediating persistence in latent infection, clinical disease, drug tolerance, treatment relapse, and heritable drug resistance however remain undefined.

Here, we provide a focused review of metabolic characteristics associated with *M. tuberculosis* persistence. We focus on metabolism because it is the biochemical foundation of all physiologic processes and a distinguishing hallmark of *M. tuberculosis*'s physiology and pathogenicity (5). In addition, it serves as the chemical interface between host and pathogen. However, existing knowledge of metabolism derives largely from physiologic contexts in which replication is the primary biochemical objective. The goal of this chapter is to review existing knowledge of mechanism- and/or model-specific and -independent metabolic features of *M. tuberculosis* persistence in which replicative quiescence often features as a key distinguishing characteristic. Such a perspective may help guide ongoing efforts to develop more efficient cures and inform on novel strategies to break the cycle of transmission sustaining the pandemic.

TERMS OF DISCOURSE

Medical evidence of clinically latent infection by *M. tuberculosis* predates current terminology by centuries. However, the terms used to describe the microbiologic features of this state have been used somewhat ambiguously. For the purposes of clarity, we have adapted the definitions offered by Gomez and McKinney (6) as follows:

Persistence

the ability of an organism to maintain infection in the face of either antibiotic treatment *or* host immunity. Its adjective form, **persistent**, describes the subpopulation of organisms that display this property regardless of its size or location.

Latency

the presence of infectious organisms despite asymptomatic lesions in the host.

Dormancy

the property of slowly- or non- dividing bacteria *in* or *ex vivo*.

Drug tolerance

a form of phenotypic drug resistance that is demonstrably independent of specific mutations or alleles that confer that resistance. This phenotype is reversible (not genetically encoded), and can occur independently of dormancy.

These definitions emphasize the potential diversity of contexts and mechanisms in or by which persistence can arise. For the purposes of the current discussion, we take a clinical perspective in which persistence arises in 2 distinct contexts, host immunity and chemotherapy. Both share the ability to slow or arrest *M. tuberculosis*'s replication but act through a diverse and heterogeneous set of selective pressures whose overlap (or lack thereof) with one another remains unresolved, and are thus considered as 2 *functionally* distinct classes.

In discussing persistence, it is equally important to highlight a commonly overlooked distinction between metabolism and nutrition. Though often used interchangeably, metabolism describes the entirety of chemical reactions used to maintain the living state of cells and organisms while nutrition refers to the subset of processes used to fuel metabolism. In the context of persistence, this distinction is important because it recognizes a broader range of specific physiologic roles for metabolism beyond that of a central warehouse of precursors and energy, or conduit to the extracellular environment. Indeed, recent work has identified a growing number of cell intrinsic metabolic functions that are dissociated from uptake of nutrients in the extracellular environment (7–10). Metabolism thus serves cellular physiology in ways that are as qualitatively as quantitatively specific.

MODELS

Experimental evidence for persistence in *M. tuberculosis* infection was first reported by McDermott who showed that mice infected with *M. tuberculosis* and effectively cured with chemotherapy (as measured by recovery of colony forming units from the entire carcass of infected animals) harbored viable bacilli that could be unmasked (or re-activated) only upon immunosuppression (11). Numerous studies had previously reported the presence of visible bacteria in tuberculous lesions of humans after months of chemotherapy. However, few, if any, had recovered viable organisms. McDermott and colleagues further demonstrated that the recovered bacilli remained susceptible to the very same antibiotics used to achieve cure and had not arisen from the acquisition of resistance alleles (11). Indeed, Ford and colleagues subsequently showed that heritable drug resistance was linked to basal mutation rates, rather than pre-exposure to antibiotics (12). McDermott's work thus not only demonstrated that persistence could be mediated by a subpopulation of dormant organisms but that this subpopulation could also survive the joint pressure of host immunity and antibiotic selection. In support of this view, work by Gill *et al.*, using a plasmid-based reporter of growth rate, recently showed that organisms recovered from the chronic (or persistent) phase of infection in mice exhibited slowed, but measurable, rates of growth despite steady titers (13).

The issue of persistence in human disease was first raised by WW Stead who questioned whether new disease occurred because of reactivation of a clinically latent (or contained)

infection or due to a new infectious event (14). If reactivation were simply the result of a secondary infection acquired by proximity with an infectious host, there was no reason to believe that persisters were anything more than dying *M. tuberculosis*, incapable of causing disease. However, population-based epidemiologic studies, combined with molecular strain typing methods, showed that disease relapse in countries with low burden was largely due to the same strain causing the initial episode of disease, suggestive of reactivation (15–18), while relapse in high burden areas was associated with different strains, suggestive of re-infection (19, 20). Work by Vandiviere, comparing bacilli recovered from resected lesions of patients receiving chemotherapy that were either in communication with (open) or closed off (closed) from the airways, showed that bacteria recovered from closed lesions were uniformly drug susceptible, while those from open lesions were drug resistant despite similar levels of drug penetration (21). Clinical studies conducted by the British Medical Research Council subsequently showed that the duration of TB chemotherapy could be shortened by the inclusion of drugs, such as rifampicin and pyrazinamide, that exhibited activity against non- or slowly replicating *M. tuberculosis* cultures *in vitro* (22, 23). These findings thus not only demonstrated that persistence was a feature of human tuberculosis but was also mediated by a subpopulation of *M. tuberculosis* refractory to sterilization by host immunity and conventional chemotherapy.

Prior to recognition of persistence in latent infection or chemotherapy, *M. tuberculosis* was long noted for its *in vitro* resilience to a broad range of environmental stresses. Indeed, seminal work by Corper and Cohn showed that clinical *M. tuberculosis* isolates could not only survive in sealed culture vessel for 12 years at 37°C but also retain their virulence (24). *M. tuberculosis* exposed to host- or chemotherapy-derived stresses have since served as useful *in vitro* models of persistence. Within humans, *M. tuberculosis* occupies a dynamic and heterogeneous range of intra- and extra-cellular microenvironments, each of which includes multiple biochemical conditions capable of restricting its replication *in vitro*. Known stringencies include nutritional and micronutritional (vitamin and co-factor) deficiencies, oxidative, nitrosative, acidic, hypoxic, and membrane-perturbing stresses (25). In addition, sublethal exposure to antibiotics themselves has been reported to slow *M. tuberculosis*'s replication and induce persistence to the same and other antibiotics (26). *In vitro* models have thus enabled detailed mechanistic studies of persistence. However, fundamental uncertainties concerning the location of host- and drug-induced persistent *M. tuberculosis*, and heterogeneity of conditions encountered therein, have hindered efforts to establish physiologic relevance.

In a practical illustration of the potential dissociation between *in vitro* and *in vivo* studies, Pethe *et al.* reported the results of a drug screen that focused on a class of pyrimidine imidazoles with strong *in vitro* efficacy but no antitubercular activity in the murine model (27). Investigating the cause of this dissociation, the authors discovered that the *in vitro* activity of these compounds required the inclusion of glycerol in the culture medium. Studies of *M. tuberculosis* mutants unable to metabolize glycerol (due to deletion of the activating enzyme, glycerol kinase) in mice however failed to reveal a survival defect. This finding thus highlighted an important, but previously unrecognized, limitation of commonly used culture conditions that had been selected for their ability to promote maximal *in vitro*, but not *in vivo*, growth (28).

Recognizing the physiologic complexity of the host niche, studies of persistence have also made use of cell culture-based models. Macrophages are the primary cell type infected by *M. tuberculosis* and constitute the largest cellular reservoir of *M. tuberculosis* during the establishment and maintenance of chronic infection (29). Within macrophages, *M. tuberculosis* resides chiefly within the phagosomal compartment where it is often able to persist for days *in vitro* and years *in vivo* (30, 31). At the same time, *M. tuberculosis*-containing phagosomes have long been noted for their heterogeneity with respect to their biochemical composition and ability to restrict *M. tuberculosis*. For example, nascent phagosomes, while hypoxic and nutrient poor, are capable of slowing but not suppressing *M. tuberculosis* replication, while fusion with endomembrane compartments and immune activation by cytokines enables the host to suppress, and sometimes even kill, *M. tuberculosis* by altering its chemistry to include acidic pH, lytic (or cell wall perturbing) enzymes and/or antimicrobial peptides, oxygenated lipids, and reactive oxygen and nitrogen species (32, 33). Recent studies have suggested that *M. tuberculosis* can even escape the phagosome to reside in cytosol (34). Given this potential range of heterogeneity, it is not surprising that the utility of these models has been defined by the type, differentiation program, and immune activation status of cells used, each of which can influence the nature and degree of *M. tuberculosis* persistence.

The largest and most complex model of *M. tuberculosis* persistence, short of humans, is the experimentally infected animal. Inbred mice are the historically oldest and most extensively studied model of TB used. While murine models of TB fail to fully recapitulate the pathology of both latent and active human TB, the availability of various inbred strains, reagents, and genetically altered mutants, and the ease with which mice can be infected and analyzed have allowed for the clear identification of genes associated with *M. tuberculosis*'s pathogenicity (35). In addition, experimental infections of other animal models with genetically altered *M. tuberculosis* mutants have been performed only rarely. In the most commonly used aerosol model of pulmonary TB in C57/B16 mice, *M. tuberculosis* persists at a stable bacterial burden, following the onset of adaptive immunity, that was found to represent a state of balanced growth and death in which the replication rate of growing organisms was approximately 20% of that observed during the initial establishment of infection and unrestricted *in vitro* growth (13). As with cell culture models, the availability of genetic mutants and biochemical and immunological reagents has made it possible to identify host and bacterial determinants of persistence. In a recent application of this approach, Liu *et al* identified specific determinants of host immunity associated with drug-induced persistence (36). Nonetheless, the growing use of other animal models such as the guinea pig, rabbit, and non-human primates has begun to yield important insight into those aspects of TB pathogenesis not modeled by the mouse.

METHODS

Despite over 50 years of research, studies of persistence have been hindered, in part, by fundamental uncertainties regarding the heterogeneity, size and location of clinically relevant persisters; and their relationship to the multiplicity of potential mechanisms described above. Indeed, while bacterial numbers during latent infection and following chemotherapy often reach undetectable levels, clinico-pathologic studies have

demonstrated large bacterial burdens at the center or acellular rim of necrotic or cavitary lesions that are often hypoxic and rank among the strongest known risk factors for treatment relapse (37). As a result, studies of persistence have been heavily influenced by the technical bias of available experimental methods and their compatibility with existing models. This bias initially favored the study of deterministic factors associated with persistence of majority subpopulations of *M. tuberculosis*. However, recent advances in single cell technologies in combination with molecular genetic approaches have expanded this scope to enable the discovery of stochastic factors and minority subpopulations (38–40).

Agar-based culture methods have long served as the gold standard measure of persistence. Pre-dating the recognition of persistence as a pathophysiologic trait, these methods provided the first evidence of *M. tuberculosis*'s unusually robust tolerance to a wide range of *ex vivo* stresses. Agar-based methods subsequently enabled the foundational discovery of persistence as a microbiologic trait and have since served as the chief modality for its detection and enumeration. Interestingly, recent work has suggested that, despite their historical significance, these methods may fail to detect a significant proportion of viable organisms. Such organisms have been found to be selectively recoverable from liquid- but not agar-based media, suggesting the existence of yet additional persistent subpopulations (41). From a broader perspective however, the inherent requirement for growth under artificial conditions has restricted the utility of all culture based methods outside of detection and enumeration to *in vitro* settings.

The advent of nucleic acid-based technologies subsequently enabled systems-level studies of *M. tuberculosis* incubated under conditions thought to represent the persister niche *in vitro* and *ex vivo*, as well as *M. tuberculosis* recovered from its native milieu in the native lung of infected animals and humans. These technologies initially served to provide descriptive transcriptional profiles but were soon followed by functional methods that enabled the identification of specific genes required for persistence. The latter methods specifically entailed competitive negative selection assays comparing an input pool of transposon mutants to the pool recovered after some stress (42), and found application in *in vitro* and animal models of host or drug-induced persistence where bacterial biomass at physiological loads is limiting (43–48). The impact of these methods however was somewhat tempered by their general dependence on the accuracy and completeness of their accompanying bio-informatic annotations and limited coverage of genes also required for optimal *in vitro* growth. Limitations notwithstanding, these approaches have provided broad qualitative insights into *M. tuberculosis* persistence as manifest *in vitro*, and in macrophages, animal models and humans.

Advances in analytical chemistry, mass spectrometry and NMR have recently enabled conceptually analogous systems-level biochemical studies. These technologies have thus far contributed specific insight into the *in vitro* proteome, *in situ* metabolome, and *in vivo* metabolome of persistent *M. tuberculosis* recovered from or resident to *in vitro* culture systems, macrophages and the lungs of infected animals (49–55). Owing chiefly to limitations in sensitivity however, these approaches have been primarily restricted to models in which persistence is manifest as a deterministic property of the majority population of *M. tuberculosis* present.

While direct studies of persistence mediated by minority subpopulations and/or stochastic mechanisms were once refractory to traditional experimental technologies, this hurdle has also been recently overcome by advances in robotic high throughput screening and single cell imaging technologies. These technologies have specifically enabled the discovery of a form of chemotherapeutically induced persistence in which *M. tuberculosis* is able to withstand sterilization by the frontline drug isoniazid through the temporally stochastic extinction of transcription of its activating enzyme, catalase(38). High throughput robotics have similarly enabled the screening of vast chemical libraries for compounds active against non- or slowly replicating *M. tuberculosis* populations (56–58).

MEASUREMENTS

Focusing on metabolism, existing knowledge of persistence derives from a mosaic of experimental models and methods of varying physiologic relevance. Such uncertainties have made it difficult to relate the findings from one experimental setting to another as well as to the physiologically distinct forms of persistence observed *in vivo*. In the case of metabolism, this challenge has been further complicated by the fact that metabolic enzymes serve pathways that are often organism- and/or condition-specific and thus functionally unannotated.

Host-induced persistence

Limitations notwithstanding, evidence for a number of model-specific and –independent characteristics has slowly begun to emerge. Chief among these are those pertaining to respiration. Though classified as a strict aerobe, *M. tuberculosis* is able to survive within intra- and extracellular niches that are either directly or functionally limiting for oxygen. Indeed, direct biochemical measurements have reported oxygen concentrations within mouse and human macrophage phagosomes as low as 1% while those of *M. tuberculosis*-containing lesions in mice, rabbits and non-human primates were generally less than 5% (59–62). Oxygen concentrations aside, *M. tuberculosis* also encounters nitric oxide (NO) as an inflammatory product of immune activated macrophages, resulting in a functional poisoning of its respiratory chain. At the same time, auto-oxidation of NO also gives rise to nitrate, which can serve as an alternate terminal electron acceptor, second only to molecular oxygen. Interestingly, *M. tuberculosis*'s ability to respire nitrate has been found to accompany a transcriptionally-mediated program that is activated upon gradual oxygen depletion to levels below 1%, and facilitate survival in response to mild acid stress (pH= 5.5) similar to that achieved upon immune activation (60). *In vitro* studies have further shown that reductions in oxygen concentration below 1–2% and availability of nitrate are also linked to marked decreases in drug susceptibility. Respiration has thus attracted specific interest as a metabolic feature of persistence in the setting of both natural infection and chemotherapy.

Microbiologic studies first demonstrated that while *M. tuberculosis* is unable to survive abrupt transfer from vigorous aeration to anaerobic conditions *in vitro*, it is able to survive for extended periods of time at oxygen tensions as low as 0.28 mm Hg (0.03%) if allowed to adapt to gradual oxygen depletion (63). As oxygen depletes, *M. tuberculosis* first reduces its

respiratory rate at a dissolved oxygen concentration of ~6% though growth continues unabated (64). At oxygen concentrations of ~1–2% (non-replicating persistence, or NRP-1), growth slows with an arrest of DNA synthesis and decrease in ATP levels, with increase in levels of a glycine dehydrogenase-like activity (63). Consistent with these changes, transcriptional profiling studies of *M. tuberculosis* subjected to hypoxia, NO, or recovered from infected macrophages and mice have shown increased expression of a Type II (non-proton translocating) NADH dehydrogenase and less energy-efficient, but higher oxygen-affinity, cytochrome *bd* oxidase, and decreased expression of ATP synthase (65–67). Genetic deletion of the cytochrome *bd* oxidase has been further shown to impair survival of *M. tuberculosis* during transition to the chronic (or persistent) phase of infection(65). As oxygen depletes further (typically below 0.06%; or NRP-2), both DNA and RNA synthesis arrest and glycine dehydrogenase activity decreases, while *M. tuberculosis* resides in a physiologically quiescent state as manifest by the synchronized resumption of RNA synthesis immediately upon re-aeration and delayed re-initiation of DNA replication until after completion of the first division (68).

Interestingly, adaptation to 1% oxygen, sublethal concentrations of NO, and residence within macrophage cell cultures and mice, are all accompanied by a transcriptional increase in expression of the nitrate transporter, *narK2* (69). This increase in expression has been further shown to be accompanied by a biochemical increase in nitrate respiration. These findings thus support the physiologic relevance of nitrate as a prevalent feature of the hypoxic niches occupied by *M. tuberculosis*, independent of its specific production as an auto-oxidation product of NOS2 which requires oxygen as a substrate and loses 80–90% of its activity at 1% oxygen (70). Moreover, recent work has shown that respiration of nitrate gives rise to nitrite that alters *M. tuberculosis*'s physiology through mechanisms independent of its potential dismutation back into nitric oxide and nitrate. These include inhibition of ATP consumption, oxidation of iron from the ferrous to ferric state, disrupting iron sulfur clusters and perhaps contributing to resistance to the frontline drug isoniazid (by potentially suppressing its activation by the heme-containing catalase), and inhibition of growth upon aeration (60).

From a metabolic perspective, ¹³C tracing studies conducted in 2 independent studies have shown that the foregoing changes in respiration are linked to accompanying alterations in the structure of its TCA cycle (71, 72). As oxygen depletes to ~1%, *M. tuberculosis* increases expression of the glyoxylate shunt enzyme isocitrate lyase to produce large amount of succinate. This increased production of succinate is hypothesized to enable *M. tuberculosis* to flexibly sustain membrane potential, ATP synthesis, and anaplerosis at a rate proportional to its respiratory capacity; such that some of the unused excess can be secreted to maintain membrane potential while the remainder is stored to facilitate immediate resumption of carbon flow and ATP synthesis upon re-aeration. Accordingly, provision of nitrate at 1% oxygen abolished succinate secretion and restored TCA cycle activity, ATP levels, and NADH/NAD ratios to near aerobic levels (72). As oxygen levels deplete further, *M. tuberculosis* has been shown to upregulate expression of genes of the reductive arm of its TCA cycle with an accompanying increase in reductive half-cycle activity which also produces succinate as an endproduct (71, 72). In this setting, the near neutral midpoint potential of the succinate/fumarate redox couple ($e^{0'} = +0.03$ V) makes it suitable to

accumulate as a fermentation product of fumarate reductase and facilitate redox balance. Generation of succinate thus appears to serve as a type of multifunctional “metabolic battery” capable of flexibly sustaining membrane potential, ATP synthesis, and TCA cycle precursors and functioning as a biochemical bridge between oxidative and fermentative metabolic states.

In a more functionally-oriented approach, Hartman and colleagues characterized the impact of genetically deleting the membrane anchor subunit of *M. tuberculosis*'s succinate dehydrogenase (*sdh1*; *rv0249c*) (64). *sdh1* has dual roles in the TCA and the respiratory chain where it oxidizes succinate to fumarate and delivers two electrons to the membrane electron carrier menaquinone. Accordingly, isotope labeling studies on *M. tuberculosis* strains harboring clean deletions of *sdh1* confirmed that succinate oxidation was diminished during aerobic growth, but continued uncontrolled when shifted into an anaerobic environment. This mismatch between succinate metabolism and respiration resulted in a tenfold decrease in bacterial titers in the lungs of mice that developed severely hypoxic lesions.

Arguably, the most compelling line of evidence in support of hypoxic respiration as a critical feature of host-induced persistence however is the clinical efficacy of bedaquiline (BDQ) and pretomanid (PA-824) (and its related nitroimidazo-oxazole, Delamanid (OPC-67683)) in human studies (73–75). BDQ is a potent inhibitor of *M. tuberculosis*'s ATP synthase that binds to the membrane-embedded rotor (c ring) and prevents the shuttling of Na⁺ and/or H⁺ ions needed to power ATP synthesis (76). Moreover, microbiologic and animal studies indicate that BDQ is capable of killing both replicating and non-replicating *M. tuberculosis* as modeled *in vitro* by hypoxia and nutrient starvation, and *in vivo* in an established model of chronic infection, wherein activity manifest as an accelerated bactericidal activity (77–81). PA-824, in contrast, is a bicyclic nitroimidazole compound whose activity against hypoxic *M. tuberculosis* is mediated by intrabacterial release of NO by a deazaflavin-dependent nitroreductase (Ddn; Rv3547) (82, 83). However, like BDQ, PA-824 was also found to exhibit activity during the continuation phase of chemotherapy in a high dose aerosol challenge model of TB thought to model persistent *M. tuberculosis in vivo* (84, 85), as well as in patients with smear-positive disease (86, 87). Taken together, these findings unequivocally establish hypoxic respiration as a clinically relevant feature of *M. tuberculosis* persistence.

Respiration aside, knowledge of metabolic adaptations specific to host-induced persistence remains considerably more model-specific and dependent on bioinformatically-derived inferences. These inferences derive chiefly from gene expression and mutant analysis studies of *M. tuberculosis* (i) incubated under host-relevant *in vitro* conditions thought to model persistence; or (ii) recovered from the chronic phase of infection in animals and humans.

Studies of the former category have specifically examined nutrient starvation (modeled by incubation in phosphate buffered saline), acid pH (approximating those of resting and immune activated macrophage phagosomes), bacteriostatic concentrations of nitric oxide (as described above), and a multi-stress model consisting in 5% oxygen, 10% CO₂, pH=5, and 10% Dubos medium. Interestingly, exposure to both nutrient starvation identified a

generalized downregulation of genes associated with aerobic respiration suggestive of metabolic changes similar to those associated with hypoxia (described above) (88). These included downregulation of subunits of its Type I NADH dehydrogenase and ATP synthase and upregulation of its fumarate reductase and isocitrate lyase, while expression of annotated genes of central carbon metabolism were generally reduced, and oxygen consumption rates were significantly reduced.

Exposure to acid pH identified a surprisingly small number of annotated genes of metabolic function, of which only upregulation components of the ESX-1 secretion system was observed in an independent study (89, 90). Nonetheless, a comparison of *in vitro* acid stress (pH= 7 vs. 6.5 vs. 5.5) to infection of bone marrow-derived murine macrophages treated (or not) with concanamycin A, a specific inhibitor of the vacuolar ATPase mediating phagosomal acidification, identified an increase in expression of acid-specific genes involved in lipid metabolism (*lipF* - Rv3487c, *whiB3* - Rv3416) and polyketide biosynthesis (*papA1* - Rv3824c, *pks2* - Rv3825c, *pks3* - Rv1180) (89); though none of the latter set of genes was found to be essential for chronic phase survival in the murine lung (91).

Exposure to the complex stress described above identified related alterations in expression of genes associated with nutrient starvation, hypoxic adaptation, and lipid metabolism (*nuoB*, *ctaD*, *qcrC*, *atpA*, *atpD*), similar to those observed in bacteria recovered from sputa from patients prior to treatment (92, 93). Other microarray-based transcriptional profiling studies in mice and humans during the chronic (or persistent) phase of infection have similarly reported alterations in expression of genes involved in central carbon metabolism and/or respiration (*aceA*, *narK2*, *nuo*, *nadC*, *menA*, *lld2*, *ppdK*), and lipid metabolism (*aceA*, *echA15*), similar to those observed in *in vitro* models. However, the overlap of specific genes identified in all models has been small and primarily limited to those involved in respiration (67, 94, 95).

Studies of the latter category have primarily emerged from the use of genetically inbred mice. Such studies were initially restricted to the identification of metabolic genes dispensable for *in vitro* growth and/or survival, but have since been expanded to include genes required for *in vitro* growth thanks to the advent of conditionally regulated gene expression systems (96). Together, these approaches have enabled a systematic inventorying of metabolically annotated genes required for persistence.

The first such gene to be identified was *icl1*, one of *M. tuberculosis*'s 2 annotated isocitrate lyases (ICL) (97). ICL is a canonical enzyme of the glyoxylate shunt, a pathway required for assimilation of even chain fatty acids. Deletion of *icl1* was specifically found to attenuate *M. tuberculosis* survival during the chronic, but not acute, phase of infection in mice, and during infection of interferon gamma-activated, but not resting, bone marrow derived macrophages. Based on these findings, ICL was interpreted to be essential for persistence due to its role in metabolism of even chain fatty acids. However, subsequent work showed that *icl1* also encoded activity as a methylisocitrate lyase (MCL), an enzyme involved in metabolism of the odd chain fatty acid propionate through the parallel methylcitrate cycle (98, 99). Moreover, studies of *M. tuberculosis* strains lacking both ICLs revealed a bactericidal vulnerability to both even and odd chain fatty acids that could be genetically complemented

by *icl1* alone. This vulnerability was ultimately shown to be due to loss of MCL, rather than ICL activity (99, 100). These studies thus raised the possibility that the persistence defect of ICL deficient *M. tuberculosis* observed in mice might reflect an inability to metabolize propionate instead of, or in addition to, acetate. Efforts to resolve this ambiguity however were complicated by the discovery of 2 additional physiologic roles for ICL unrelated to fatty acid metabolism altogether, adaptation to hypoxia (discussed above) and antibiotic tolerance (discussed below) (9, 101).

A second example that emerged with the use of conditionally regulated mutants was the discovery of *pckA*, a gene annotated to encode the enzyme phosphoenolpyruvate carboxykinase which catalyzes the first committed step in gluconeogenesis. Transcriptional silencing of *pckA* during the chronic (or persistent) phase of an aerosol infection in mice led to its clearance (102). In conjunction with evidence for the importance of fatty acid metabolism, these data implicated an essential role for gluconeogenesis in mediating persistence. However, this survival defect could not be recapitulated when culturing *pckA*-deficient *M. tuberculosis* on fatty acids or in resting or immune activated bone marrow derived macrophages. Thus, like the case for ICL, these studies identified *pckA* as a clear determinant of persistence but failed to reveal its specific metabolic role, owing to limitations in the accuracy and completeness of bio-informatic annotations and *in vitro* biochemical studies. Indeed, follow on biochemical studies demonstrated that *M. tuberculosis*'s PckA could catalyze the reverse reaction in an anaplerotic, rather than gluconeogenic, direction under reducing conditions such as those associated with hypoxia (103). Experimentally-based computational models of metabolic flux have similarly suggested that *M. tuberculosis* PckA may operate in an anaplerotic direction during intracellular growth within a THP-1 macrophage-like cell line (55).

Studies of additional gene deletion mutants in central carbon metabolism have similarly identified essential, but incompletely defined, roles for 2 glucokinases (*ppgK*, *glkA*), fructose biphosphate aldolase (*tba*), lipoamide dehydrogenase (*lpd*), and 2-hydroxy-3-oxoadipate synthase (*hoas/kgd*) (8, 104–106). However, like the case for *icl1/2* and *pckA*, interpretation of their specific metabolic roles in persistence remains unresolved. Glucokinase, for example, catalyzes the specific phosphorylation of glucose into glucose phosphate which can serve to biochemically trap or retain glucose within the cell, fuel glycolysis and/or facilitate the generation of NADPH. Fbp can similarly serve both glycolysis and gluconeogenesis, while Lpd was shown to serve as a subunit of *M. tuberculosis*'s canonical pyruvate dehydrogenase complex, peroxynitrite reductase and branched chain ketoacid dehydrogenase. Recent studies similarly demonstrated that HOAS/Kgd was competent to support production of 3 products, 2-hydroxy-3-oxoadipate, succinate semialdehyde, and succinate, but found to mediate defense against glutamate toxicity and reactive nitrogen intermediates when present in *M. tuberculosis* cultured under standard *in vitro* conditions (106).

Gene deletion studies have also identified specific roles for the core proteasome subunits (*prcA* and *prcB*), the stringent response regulator (*relA*), the methionine biosynthetic enzyme homoserine transacetylase (*metA*), and a mycolic acid cyclopropane synthetase (*pcaA*) in persistence in mice (107–111). Both *prcBA* and *relA* have been reported to

facilitate adaptation to *in vitro* nutrient limitation in *M. tuberculosis* through roles in amino acid recycling and biosynthesis, respectively. Interestingly, however, expression of a hydrolase null allele of RelA, still competent to synthesize the stringent response alarmone (p)ppGpp, was found to be competent to complement the persistence defect of an isogenic *relA*-deficient strain in mice (112). Expression of a proteolytically null active site mutant was similarly shown to be sufficient to complement both the *in vitro* susceptibility to nitric oxide and *in vivo* persistence defects of *prcBA*-deficient *M. tuberculosis*, though complementation of other *in vitro* persistence defects associated with prolonged stationary phase incubation and nutrient starvation required the wild type allele (107). The specific metabolic functions served by PrcBA and RelA, and ways in which they facilitate persistence thus await further study. In contrast, the persistence defects of *metA* and *pcaA*-deficient *M. tuberculosis* appear more straightforward. MetA catalyzes the first committed step in methionine biosynthesis. Deletion of *metA* results in a rapid bactericidal death *in vitro* and in both the acute and chronic phases of mouse infection, the former of which can be chemically complemented with methionine supplementation alone (110). These results thus identify a likely essential role for methionine or methionine-derived metabolites in persistence. The function of *pcaA* is perhaps even more physiologically specific in that it encodes an enzyme required for the formation of the proximal cyclopropane ring of *M. tuberculosis*'s immunoreactive alpha-mycolic acids, loss of which was accompanied by alterations in cording morphology (a biomarker of virulence), lipid profile and cytokine responses (111, 113, 114). These findings thus suggest that *pcaA* may facilitate persistence through effects on the composition and immunoreactivity of *M. tuberculosis*'s cell surface lipids.

Based on the foregoing considerations, it is interesting to reconsider historical evidence implicating fatty acid oxidation as an essential feature of persistence. The first line of evidence emerged from experimental studies by Segal and Bloch who found that *M. tuberculosis* isolated *ex vivo* were primed to respire on fatty acids (115). This view was reinforced by the subsequent publication of its genome which revealed over 250 enzymes involved in fatty acid metabolism, including 82 annotated as either fatty acid desaturases or acyl-CoA dehydrogenases (116). These numbers were interpreted to indicate extensive redundancy in utilization of fatty acid substrates, though only 2 of *M. tuberculosis*'s over 100 β -oxidation genes have been determined to be essential *in vitro* (117). More specific, though circumstantial, evidence in support of a role for fatty acid metabolism in human infection emerged with the discovery of lipid droplets within *M. tuberculosis* recovered from the sputum of patients with active tuberculosis. These droplets were found to contain host-derived triacylglycerol (TAG) (92) that could be mobilized when respiration was inhibited, presumably as a carbon and energy source (118). In addition, similar lipid inclusions could be modeled *in vitro* following incubation of *M. tuberculosis* in hypoxia (63). However, the strongest and perhaps most influential, evidence in support of the essentiality of fatty acid utilization in persistence stemmed from the discovery of a selective survival defect of *icl* *M. tuberculosis* during the chronic, but not acute, phase of infection in mice, whose interpretation was discussed above (97). Thus, while likely to be a feature of its metabolism during infection, direct evidence for the essentiality and nature (whether fueled by intra- or extracellular lipids) of fatty acid metabolism in persistence remains surprisingly scant.

Ambiguities notwithstanding, nucleic acid-based technologies have also enabled empiric genome-wide inventorying of metabolic genes required for persistence. The most comprehensive of these inventories was reported by Sasseti and Rubin who made use of transposon-mutagenized libraries of *M. tuberculosis* to identify genes required for survival in resting and immune activated macrophages, and in the spleens of mice infected by tail vein injection (91). These studies specifically enabled the identification of genes with competitive fitness defects in the acute and chronic phases of infection (TABLE 1).

Among the latter, work by Griffin and Sasseti identified a gene cluster required for import of host cholesterol (44). This finding launched a litany of follow on genetic and biochemical studies showing that *M. tuberculosis* could both import and catabolize cholesterol, and that this activity was essential for persistence in the lungs of chronically infected animals and for growth within the IFN- γ -activated macrophages (119–121). Cholesterol that is metabolized in *M. tuberculosis* (when used as a sole carbon source) is cleaved into pyruvate and propionyl-CoA and/or acetyl-CoA during aerobiosis. The resulting pyruvate can be oxidized into the TCA cycle. However, the propionyl-CoA that is generated must first be detoxified primarily by the methylcitrate cycle (less so by the methylmalonyl pathway) into succinate and pyruvate before entering central carbon metabolism (120), while the carbon of the side chain of cholesterol can be assimilated into both PDIMs and sulfolipid-1 (SL-1). The physiologic role and specific enzymes required for cholesterol metabolism in persistence however remain unclear as it supports only weak *in vitro* growth as a carbon source while a transposon mutant interrupted in Rv1106c (which is required for growth when cholesterol is provided as the sole carbon source) displayed no phenotype in either macrophage culture or guinea pig infection (122).

Looking beyond central carbon metabolism, gene deletion studies have identified essential roles for biosynthesis of the co-factors, NAD and biotin, in persistence. Silencing of either *nadE* or *bioA* resulted in clearance of *M. tuberculosis* from the lungs of mice when depleted during the chronic phase of infection (123, 124). NadE is a glutamine-dependent NAD(+) synthetase that catalyzes the last committed step in the nicotinamide adenine dinucleotide (NAD) synthesis pathway. NAD(+) itself has long been implicated in survival in dormancy since Wayne and Lin found that glycine dehydrogenase and ICL activities were increased in their model of dormancy (125). Silencing of *bioA* resulted in a deficiency of biotinylated proteins in *M. tuberculosis*, leading the authors to posit that fatty acid biosynthesis would be impaired in the mutant (124). The fact that depletion resulted in a lethal phenotype in the chronic phase of infection in mice may thus serve as additional evidence in support of fatty acid metabolism as an essential feature of persistence. Fatty acid metabolism notwithstanding, these findings identify NAD+ and biotin as limiting co-factors, either due to increased turnover or demand, required for persistence during the chronic phase of infection in mice.

Interestingly, recent advances in proteomics and chemical biology have begun to deliver analogous systems level biochemical insights into persistence. Such studies have thus far focused on *in vitro* models of hypoxic *M. tuberculosis*. Comparing the quantitative proteomic composition of replicating *M. tuberculosis* against NRP-1, NRP-2, and re-aerated *M. tuberculosis*, Schubert and colleagues identified specific alterations in the levels of its

respiratory enzymes (including its Type I (Nuo) and Type II (Ndh/NdhA), cytochrome c oxidase/reductase (Cta/Qcr), cytochrome *bd* oxidase (Cyd), and ATP synthase), consistent with prior biochemical, transcriptional and metabolomics studies (126). This study also identified additional linked changes in the levels of enzymes specifically involved in the acyl CoA, alanine/aspartate/glutamate, trehalose, cholesterol/lipid and quinone biosynthesis, similarly consistent with genetic studies.

In a complementary biochemical approach, Ortega and colleagues made use of a more functional approach called ‘activity-based protein profiling (ABPP) (127). ABPP specifically enables proteome-wide profiling of the *in situ* activity (rather than abundance) of a given class of proteins within a cell or cell lysate based on their vulnerability to a covalently reactive, active site-specific probe. Using a serine hydrolase-specific probe, Ortega and colleagues identified decreases in the activity of 3 enzymes involved in the biosynthesis of *M. tuberculosis*’s cell envelope mycolic acids (the antigen 85 carboxyesterases, which generate trehalose mono- and dimycolates; Pks13, an enzyme involved in phthiocerol dimycolate; and TesA, a thioesterase), in hypoxic, compared to replicating, *M. tuberculosis*, and consistent with a prior lipidomic study using the same experimental model (127, 128).

Drug-induced persistence

In contrast to host-induced persistence, comparatively little attention has been paid toward the metabolic features of drug-induced persistence. Screening a pool of signature-tagged *M. tuberculosis* transposon mutants, Dhar and McKinney identified *cydC*, a gene annotated to encode an ATP-binding cassette (ABC) transporter required for assembly of the cytochrome *bd* oxidase, as a mediator of isoniazid-induced persistence or tolerance in mice (51). Disruption of *M. tuberculosis cydC* selectively accelerated bacterial clearance in INH-treated mice without affecting growth or survival of untreated mice. This effect was specific for INH and could be genetically complemented *in vivo* with a wild type allele. However, it could not be recapitulated *in vitro* using host-like conditions capable of inducing expression of *cydC*, providing further evidence for the limitations of *in vitro* systems alone. Notwithstanding, it is interesting to note that, as mentioned above, *narG*, another alternate respiratory chain component, was also found to mediate isoniazid tolerance *in vitro* (60).

In a study examining the relationship between antibiotic tolerance and bacterial growth rate, Baek *et al.* identified *tgsI* in a transposon mutant screen for genes whose disruption resulted in a growth or survival advantage under hypoxia (129). *tgsI* is a well characterized triglyceride synthase that constitutes the dominant Tgs activity under hypoxia. Moreover, targeted deletion of *tgsI* was shown to prevent triacylglyceride synthesis and redirect carbon to increase flux through *M. tuberculosis*’s TCA cycle. This increase was subsequently shown to confer an increase in susceptibility of *M. tuberculosis* to a broad panel of antibiotics under conditions of *in vitro* hypoxia and in mice. It was thus proposed that redirection of metabolic flux away from the TCA cycle and towards the synthesis of triglycerides served as a mechanism of antibiotic-induced persistence (or tolerance) in hypoxic *M. tuberculosis*. Shi *et al.* similarly proposed a model in which carbon and nitrogen

are repurposed into TAG and glutamate for use during growth arrest and regrowth once favorable conditions were re-encountered (130).

Work by Nandakumar *et al.* more recently identified *M. tuberculosis*'s isocitrate lyases as a similarly broad metabolic mediator of antibiotic tolerance in replicating *M. tuberculosis* (101). This work specifically showed that: (i) 3 mechanistically unrelated clinical antibiotics (isoniazid, rifampicin and streptomycin) all triggered an increase in expression of *M. tuberculosis*'s ICLs, in a carbon source-independent manner; (ii) absence of ICL activity led to a significant increase in susceptibility to all 3 antibiotics; (iii) this heightened susceptibility could be functionally complemented with a chemical anti-oxidant. This study thus suggested that *M. tuberculosis*'s ICLs mediated antibiotic-induced persistence (or tolerance) by serving as a form of anti-oxidant defense in which increased flux through the glyoxylate shunt mitigates against the production of respiratory radicals arising from the generation of NADH in the oxidative arm of its TCA cycle.

Awaiting further studies, it is becoming apparent that, like its host-induced counterpart, antibiotic-induced persistence is an active cellular process, rather than passive consequence of slowed or arrested growth, likely to be encoded by specific metabolic pathways.

MESSAGES

Accumulating evidence has left little doubt about the importance of persistence or metabolism in the biology and chemotherapy of tuberculosis. However, knowledge of the intersection between these two factors has only recently begun to emerge. The goal of this chapter has been to reframe our understanding of each with respect to the other, their relationship to the fundamental biology of *M. tuberculosis*, and the potential union of these relationships to identify novel potential strategies against the pandemic.

One theme to emerge from this synthesis is the need for continued, but regulated, electron transport chain activity; a finding common to studies of persistence in other bacterial species. In *M. tuberculosis*, strains with deletion or inactivation of several individual components of the respiratory chain and glycolysis are attenuated by one log in the murine lung. Additionally, two of the most recent antitubercular drugs - Bedaquiline and Pretomanid - target the ATP synthase and respiratory chain and have activity during the chronic phase of infection. In terms of drug tolerance, this is consistent with investigations of persistence in *E. coli* by several findings including screens for factors involved in tolerance which turned up genes in central carbon metabolism and aerobic respiration (131). Other work found that metabolic stimulation of glycolysis kills drug tolerant persisters by stimulating PMF both aerobically and anaerobically (132), and that disruption of stationary phase respiration has a similar effect (133). The functional redundancy of the *M. tuberculosis* electron transport chain would complicate similar work, but the construction of conditional deletion strains offers an analytical approach to test these hypotheses in the future.

Respiration aside however, knowledge of specific metabolic processes required for persistence remains scant and heavily dependent on the use of genomic orthologies derived from organisms and ecologies distinct from those of persistent *M. tuberculosis*. Further

complicating this dependency is the modular nature of metabolic pathways and the physiologic functions they serve, which for most organisms has been limited to replication. Systems biologists have been able to define the objective function of *E. coli* in differing growth states (134). However, lacking a reliable, experimentally tractable model system, the use of such predictions is unlikely to yield insights that can be put to test.

Limitations notwithstanding, it is interesting to note that the most represented, though understudied, class of annotated functions identified in screens for genes required for persistence in mice was that of transporters. Transporters are well known for a lack of strict substrate specificity. It is thus all the more surprising that loss of individual transporters of amino acids, lipids, carbohydrates, coenzymes and inorganic ions resulted in measurable defects in persistence. Delineating the substrate specificities of these transporters thus represents a potentially fruitful, but understudied, source of drug targets in this important subpopulation.

Looking back, it has becoming clear that persistence may comprise a complex and heterogeneous set of physiologic states as potentially diverse as the conditions in which it can be found. So, just as any biochemical or thermodynamic event that disrupts the binding of an antibiotic to its target might manifest as drug tolerance, any event that limits the rate of binary fission has the potential to manifest as persistence. Knowledge of *M. tuberculosis*'s metabolic ledger in each of these states thus represents a key hurdle to overcoming the challenge of persistence.

Still, there is a notion that this heterogeneity may be unified by a common set of “persister essential” processes. In fact, the terminology that we use to classify complex traits like “persistence” and “tolerance” lends the impression that inhibition of specific pathways might be controlling these broad phenotypes. However, it remains equally possible that there are as many avenues that facilitate entry into, dwell in, and exit from persistence, as there are forms of persistence. Only further studies of core metabolic processes will allow us to resolve this ambiguity (135). However, until that time, the path forward will have to move at the pace that empirical research permits.

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Table 1

Predicted essential genes for *in vivo* survival of *M. tuberculosis* at 8 weeks that are not significantly inhibited at 4 weeks (adapted from 91).

Name	Rv#	Functional class
<i>rv2808</i>	2808	Np
<i>cobC</i>	2231c	amino acid transport and metabolism
<i>afiB</i>	3805c	capsule polysaccharide biosynthetic process
<i>rv3472</i>	3472	CHP
<i>rv0199</i>	0199	CMP
<i>rv1974</i>	1974	CMP
<i>aceE</i>	2241	energy production and conversion - pyruvate dehydrogenase E1
<i>rv0100</i>	0100	extracellular region
<i>rv0098</i>	0098	fatty acid biosynthetic process
<i>rv1021</i>	1021	general functional prediction only
<i>rv3649</i>	3649	general functional prediction only
<i>rv1939</i>	1939	general functional prediction only
<i>ctpD</i>	1469	inorganic ion transport and metabolism
<i>mce1A</i>	0169	lipid transport and metabolism – mycolic acid transport
<i>pks16</i>	1013	lipid transport and metabolism
<i>rv3523</i>	3523	lipid transport and metabolism
<i>rv3371</i>	3371	lipid transport and metabolism - triacylglycerol synthase
<i>Chp2</i>	1184c	plasma membrane - Diacyltrehalose acyltransferase
<i>ung</i>	2976c	replication, recombination and repair
<i>nnp</i>	0101	secondary metabolites biosynthesis, transport, metabolism
<i>rv2857c</i>	2857c	secondary metabolites biosynthesis, transport, metabolism
<i>pks12</i>	2048c	secondary metabolites biosynthesis, transport, metabolism
<i>rv0687</i>	0687	secondary metabolites biosynthesis, transport, metabolism
<i>rv1931c</i>	1931c	transcription
<i>rv2912c</i>	2912c	transcription
<i>proS</i>	2845c	translation

CHP=Conserved Hypothetical Protein, CMP=Conserved Membrane Protein, np = no prediction