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***Mycobacterium tuberculosis* in the Face of Host-Imposed Nutrient Limitation**

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Abstract

Coevolution of pathogens and host has led to many metabolic strategies employed by intracellular pathogens to deal with the immune response and the scarcity of food during infection. Simply put, bacterial pathogens are just looking for food. As a consequence, the host has developed strategies to limit nutrients for the bacterium by containment of the intruder in a pathogen-containing vacuole and/or by actively depleting nutrients from the intracellular space, a process called nutritional immunity. Since metabolism is a prerequisite for virulence, such pathways could potentially be good targets for antimicrobial therapies. In this chapter, we review the current knowledge about the *in vivo* diet of *Mycobacterium tuberculosis*, with a focus on amino acid and cofactors, discuss evidence for the bacilli's nutritionally independent lifestyle in the host, and evaluate strategies for new chemotherapeutic interventions.

INTRODUCTION

Interactions of bacteria with the human host are, in the vast majority of cases, beneficial for both partners (1). In fact, humans are dependent on their microbial associates for nutrition, defense, and development (1). However, a minority of bacteria use the human organism as a vessel to proliferate and spread and, as a consequence, leave behind collateral damage of varying degrees. These so-called pathogens have typically evolved to inhabit niches in the human body with little competition from their commensal counterparts (2). Many of these human pathogens are intracellular bacteria, meaning that their preferred niche of proliferation and persistence is within human cells. Intracellular pathogens invade phagocytic or nonphagocytic host cells, where they replicate in specialized phagosomal compartments or in the cytosol. After having made their way into their preferred niche, they try to benefit from host nutrients and other metabolites to satisfy their bioenergetic and biosynthetic requirements (3). The dynamic metabolic interplay between pathogen and host is essential for virulence, disease progression, and infection control.

Emerging evidence suggests that the host immune system actively depletes nutrients from the cytosol or phagosome of phagocytic cells to starve the invading microbes (3–7). Such

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processes could be collectively described as “nutritional immunity.” The first scientifically described mechanism that falls under this term was host-mediated restriction of Fe by NRAMP and transferrin (6, 8). However, more recently it became clear that the host also restricts access to other transition metals, including Mn, Zn, and Cu (9), as well as carbon (10, 11) and amino acids (7, 10, 12). For example, the mammalian enzyme indoleamine 2,3-dioxygenase (IDO) actively degrades tryptophan and is activated during pathogen infection to starve the intruders of this essential amino acid (13–15). In response to host nutrient deprivation, many human pathogens, e.g., *Legionella pneumophila* (16), *Francisella tularensis* (17), and *Listeria monocytogenes* (18), have acquired elaborate mechanisms to circumvent nutritional immunity and access essential metabolites. Such nutritional virulence mechanisms (19) allowed these pathogens to evolve into natural auxotrophs for up to 10 amino acids (16, 17), a characteristic that results in partial dependency on the host. However, it remains largely unknown what *M. tuberculosis* can and cannot scavenge from the host.

The mechanisms governing *M. tuberculosis* entry into the host, replication, and dissemination are still poorly understood. Nevertheless, based on studies with *M. tuberculosis* in macrophages and animal models, as well as *Mycobacterium marinum* in the zebra fish model, a (probably simplified) picture emerges wherein the main niche of replication of *M. tuberculosis* is the phagosome of human alveolar macrophages (20). It was suggested that during the 70,000 years of coevolution of *M. tuberculosis* with its human host (21), the pathogen has developed tactics to make a stealthy entry past the commensal barrier of the upper lungs to the lower alveolar spaces, which harbor few, if any, commensals (2, 22). In the lower alveolar space, it is thought that *M. tuberculosis* uses a masking lipid, phthiocerol dimycocerosate, to avoid the microbicidal macrophages and a recruiting lipid, phenolic glycolipid, to infect the permissive ones (2, 22). Once in their preferred niche, they replicate and, mediated by mechanisms such as the type VII secretion system ESX1, induce coordinated macrophage death and phagocytosis by new macrophages, leading to granuloma formation (23–26). This process seems to enable a tremendous expansion in bacterial numbers. To achieve this, *M. tuberculosis* must access essential elements such as carbon, nitrogen, phosphorus, and trace elements.

There are possibly three stages of *M. tuberculosis* infection that provide completely different diets to the pathogen: first, active proliferation in the macrophages; second, persistence in the granuloma; and third, possibly extracellular growth in caseating lesions. Determining nutrient availability and nutrient uptake by the pathogen during these stages is extremely challenging. The first stage can be studied to a certain extent *in vitro* (e.g., with a macrophage infection model and metabolomics); however, the difficulties in distinguishing between cytosolic and phagosomal metabolites in pathogen-infected host cells are not yet resolved. The second and third stages can only be satisfyingly studied *in vivo*. Therefore, we have to rely on indirect evidence that can be garnered from *in vivo* experiments with auxotrophic strains. In this article, we will review *M. tuberculosis* nutritional requirements and vulnerabilities with a focus on amino acids and coenzymes. We aim to summarize and discuss the current data acquired from *in vitro* studies in macrophages and *in vivo* studies in animal models, with a focus on nutrient use by the pathogen and strategies of the host to limit the pathogen’s growth. Knowledge about *M. tuberculosis*’s *in vivo* diet will help to unravel the microenvironment at different stages of infection, elucidate metabolic signaling

and nutritional checkpoints in disease progression, identify mechanisms of nutritional immunity, and most importantly, identify metabolic vulnerabilities and much needed new chemotherapeutic strategies.

M. TUBERCULOSIS'S IN VIVO GROWTH REQUIREMENTS

Lessons from Metabolomics

***M. tuberculosis* in macrophages**—Defining the nutritional environment of intracellular pathogens is technically extremely challenging in terms of the infection models and the available analytical methods. At present, the analysis of metabolic host-pathogen interactions is most easily studied using infected host cells because they represent fairly well-defined metabolic entities (3, 27, 28). The main approach is to infect human cells (e.g., monocytes or macrophages) with a virulent strain of the pathogen and measure the changes in metabolites over time. With the development of high-sensitivity small-molecule mass spectrometry, it is possible to accurately measure bacterial and host cell metabolite abundance. The advantages of this approach are high sensitivity and the simultaneous measurement of hundreds of metabolites.

Several studies have been conducted to look at metabolic changes in macrophages upon *M. tuberculosis* infection. One study used gas chromatography mass spectrometry to measure changes in metabolites in THP-1 macrophages infected with the virulent strains *M. tuberculosis* H37Rv and B36 or the avirulent strains *Mycobacterium bovis* BCG and *M. tuberculosis* H37Ra (29). This analysis indicated that in cells infected with virulent *M. tuberculosis*, the abundance of glycine, aspartate, proline, isoleucine, alanine, ornithine, threonine, cysteine, and lysine decreased, whereas glutamate, serine, and valine increased (29). However, it is unknown if these results are indicative of the nutritional environment that *M. tuberculosis* experiences in the phagosome. In nearly all studies conducted so far, metabolites were extracted from homogenized cells, an approach that does not take into account the metabolic differences of the microenvironments (e.g., cytosol versus pathogen-containing vacuole) in macrophages.

To this end, Beste and coworkers (30) performed an elegant experiment using ^{13}C -flux analysis to determine which metabolites can be taken up by *M. tuberculosis* when residing in THP-1 macrophages. This study indicated that *M. tuberculosis* is able to take up alanine, glutamate/glutamine, and asparagine/aspartate from the THP-1 cells. Consistent with this finding, Gouzy and coworkers identified specific transporters for asparagine and aspartate in *M. tuberculosis* (31, 32). *In vivo* data showed that *M. tuberculosis* does not rely on these transporters for replication in mouse organs, which is supported by the fact that *M. tuberculosis* harbors the biosynthetic machinery to produce both amino acids and that natural auxotrophs of these amino acids are absent in clinical isolates. Still, the same group demonstrated that asparagine plays a role in the pH homeostasis of *M. tuberculosis* in the phagosome via the release of ammonia by the activity of asparaginase AnsA. The *ansA* deletion strains also displayed a 1-log decrease in organ burden compared to the wild-type (WT) strain in infected mice, indicating that this process might be important *in vivo*. Taken together and based on the current literature, we know that several amino acids can be taken up by the pathogen from the host, but none of them is essential for replication and survival

in the host. This suggests that *M. tuberculosis* uses nitrogen cocatabolism for nitrogen assimilation, much like its strategy of carbon cocatabolism (33, 34).

Another limitation of the cell-line-based approach is that most of the host cells used in these assays are from cancer cell lines, which have an entirely different metabolic signature than WT cells, and therefore the metabolic environment experienced by the pathogen is different and potentially changes the microbes' own metabolic response (3, 28, 35). This problem could be prevented, for example, by using macrophages derived from peripheral blood mononuclear cells; however, the difficulty of distinguishing between cytosolic and phagosomal metabolites still remains. In more recent years, imaging mass spectrometry, such as dynamic secondary ion mass spectrometry (SIMS microscopy) and matrix-assisted laser desorption ionization imaging mass spectrometry (see reference 36 for a comprehensive review), has been used on *M. tuberculosis*-infected macrophages (32) and organ tissues (37, 38). Gouzy and coworkers were able to show, using NanoSIMS, that *M. tuberculosis* can scavenge labeled aspartate and asparagine from THP-1 cells (31, 32). In the current state, these methods are costly and mostly used to detect specific metabolites because the deconvolution of metabolite signals in the complex matrices is challenging (39). In some circumstances, isotopic labeling can overcome isobaric interference, but even these approaches will need validation on a sample-by-sample basis (39). Nevertheless, further advancements in imaging mass-spectrometry technology might soon allow the label-free detection of *in situ* metabolic profiles of pathogens within host cell vacuoles and within tissue samples.

***M. tuberculosis* in host tissue**—Metabolic changes in host tissues in response to *M. tuberculosis* infection have been measured in mice and guinea pigs. A ^1H nuclear magnetic resonance-based metabolomics profiling approach in infected mouse organs showed a significant increase in tissue concentration of several amino acids (alanine, aspartate, glutamate, leucine, lysine, isoleucine, phenylalanine, tyrosine, and glutamine) (40). However, it is unclear if these differences in metabolite concentration stem from the infected or uninfected areas in these organs, and hence they may not be indicative of the nutritional environment *M. tuberculosis* finds itself in. Somashekar and coworkers (41) showed by high-resolution magic angle spinning nuclear magnetic resonance spectroscopy that lung granulomas from *M. tuberculosis*-infected guinea pigs have elevated abundance of lactate, alanine, acetate, glutamate, glutathione, aspartate, creatine, phosphocholine, glycerophosphocholine, betaine, trimethylamine N-oxide, myo-inositol, scyllo-inositol, and dihydroxyacetone.

In summary, metabolomics approaches so far have given us some indication of the metabolic changes in host tissues in response to *M. tuberculosis* infection. However, the immediate metabolic environment of a tubercle bacillus, when it is growing within a pathogen-containing vacuole in alveolar macrophages, is still elusive, and more sophisticated methods and alternative approaches are needed to measure metabolic exchanges between the pathogen-containing vacuole, the cytosol, and bacteria.

Lessons from Auxotrophic Strains

Amino acid auxotrophies—Auxotrophy is the inability of an organism to synthesize a particular organic compound required for its growth. Early on, with the advent of molecular genetics, the use of bacterial strains with auxotrophies led to breakthrough findings about the exchange of genetic material in bacteria (42–45). Using *Escherichia coli* auxotrophic strains, Tatum and Lederberg, as well as B.D. Davis, discovered the process of conjugation (43, 46), an achievement for which Tatum and Lederberg later received the Nobel Prize in Physiology or Medicine.

Typically, auxotrophic strains are relatively easy to construct because mutants can be chemically complemented by the biosynthetic end product as long as the bacterium can transport sufficient amounts of the metabolite. Bacterial auxotrophs were also among the first mutants to be constructed when genetic tools were pioneered for mycobacteria (47, 48). Most of the auxotrophic strains constructed were attenuated in mouse models (7, 49–57), which led to the idea of using such strains as live vaccine candidates, especially because the efficacy of *M. bovis* BCG, the only vaccine available against tuberculosis (TB), is limited and in many cases not protective. Unfortunately, most of these live attenuated *M. tuberculosis* strains provided only little to no improvement in protection compared to BCG. Still, some of these auxotrophic strains have proven extremely valuable as avirulent strains of *M. tuberculosis* that can be used and manipulated under less restrictive biosafety level II conditions (58). Interestingly, despite the wealth of auxotrophic mutants, they have rarely been considered as metabolic probes *in vivo*. Arguably, an auxotrophic strain is the perfect biosensor to inform about the availability of nutrients *in vivo*. Here, we will review the literature on auxotrophic strains in the context of *in vivo* nutrition.

Aspartate and asparagines: To date, no aspartate or asparagine auxotrophs of *M. tuberculosis* have been isolated, although considerable advances have been made in understanding the role of these metabolites in *M. tuberculosis* pathogenesis. Gouzy and coworkers have inactivated the only aspartate transporter in *M. tuberculosis*, AnsP1, and shown that the mutant (*ansP1*) could not grow on aspartate as the sole nitrogen source (32). Furthermore, using NanoSIMS, these authors demonstrated that *M. tuberculosis* can scavenge labeled aspartate from THP-1 cells, and a mouse infection experiment showed growth of *ansP1* in lungs and spleens, albeit to a lower organ burden (1-log decrease) compared to the parental strain (32). The fact that the *ansP1* mutant could still replicate *in vivo* argues that aspartate is mainly produced biosynthetically by the bacterium and not taken up from the host. Moreover, the studies by Gouzy et al. suggest that the role of host-derived aspartate and asparagine in TB pathogenesis has to do with pH homeostasis rather than nutrition per se (31, 32). The asparagine transporter mutant *AansP2* was still virulent in mice, but inactivation of asparaginase (encoded by *ansA*) of *M. tuberculosis*, the enzyme that converts asparagine to aspartate and ammonium, led to a strong *in vivo* phenotype and strong susceptibility to low pH (31). It remains to be seen if host-derived aspartate and asparagine also have a nutritional role in TB pathogenesis. Constructing auxotrophic mutants and testing them *in vivo* could help to delineate their role in extracellular pH control and nutrition.

Arginine: Gordhan and coworkers constructed an *argF* mutant strain, *AargF*, by homologous recombination (55). ArgF encodes an ornithine carbamoyltransferase that converts ornithine to citrulline. Interestingly, *M. tuberculosis argF* could not be rescued by citrulline but was chemically complemented by the downstream metabolites argininosuccinate and arginine, indicating that the mutation had polar effects on *argG* (55). One interesting feature of this mutant is its rapid death and culture sterilization *in vitro* (55). *In vivo* experiments in immunocompetent DBA/2 mice or immunocompromised SCID mice showed strong virulence attenuation; however, the SCID mice still succumbed to *argF* infection, indicating that the mutant could potentially scavenge arginine from the host (55). However, evidence of complementation is absent, and colonies isolated from diseased SCID mice were not analyzed for potential suppressor mutants (55).

Macrophages have dedicated arginine transporters (e.g., SLC7A2) (59) to fuel nitric oxide (NO) production by NO synthase (iNOS) (58). Pathogen entry triggers NO production and therefore arginine depletion (59). Second, arginine concentration is controlled by arginases (Arg1 and Arg2) (58), and it is thought that arginases control arginine homeostasis to regulate NO levels (60, 61). When Arg1 knockout mice were infected with *M. tuberculosis*, the bacterial replication stopped earlier (1 log difference) than in WT mice (62). This was ascribed to an increase in NO production in the absence of arginine-degrading arginase 1 (62). To our knowledge, it is unknown if arginine depletion is an antibacterial mechanism or if it is just a side effect of antibacterial NO production and homeostasis. Hence, it would be interesting to test if an *M. tuberculosis* arginine auxotroph can grow in an iNOS/Arg1 double-knockout mouse that theoretically loses its ability to lower arginine concentrations.

Cysteine: Senaratne and coworkers constructed a cysteine auxotrophic strain by deleting *cysH* from *M. tuberculosis* (63). CysH encodes a phosphoadenosine phosphosulfate (PAPS) reductase that converts PAPS to sulfite and is involved in sulfur assimilation. The *cysH* deletion mutant (*cysH*) was attenuated in immunocompetent mice but not in immunocompromised animals, suggesting that chemical complementation of this auxotroph is dependent on the adaptive immune system. Both methionine and cysteine complemented the mutant *in vitro*, but the nature of the *in vivo* complementation is still unknown. Given the early position of CysH in the sulfur assimilation pathway, it is possible that this mutant is able to scavenge sulfide, taurine, or cysteine from the host. Methionine was long believed to be an amino acid that could be scavenged from mouse tissue, but recently we showed that this is not the case (57). Taken together, the current literature suggests that *M. tuberculosis cysH* can scavenge a yet unknown sulfur-containing compound from the host other than sulfate.

Methionine: Methionine auxotrophy was among the first auxotrophies described in mycobacterial research. Two transposon mutants of *M. bovis* BCG that could grow in the presence of methionine, but not with sulfide or cysteine, were isolated and shown to survive in mice just as well as the WT strain (47, 64). This led the authors to hypothesize that methionine can be scavenged from the host. The studies showed that the transposon insertions were in genes encoding for the proteins SubI and CysA, both subunits of the sulfate uptake permease (31).

Contrary opinions exist about the ability of mycobacteria to scavenge cysteine from the medium. Whereas it has been corroborated that a *cysA* mutant of BCG could not be complemented by cysteine, a *cysH* mutant of *M. tuberculosis* could be complemented. It is possible that there are species-specific differences in cysteine biosynthesis or that differences in experimental setups led to this discrepancy. However, what both mutants had in common was their ability to grow normally in the presence of methionine. Methionine biosynthesis is linked to sulfur metabolism by transsulfurylation, which is catalyzed by the two enzymes cystathione γ -synthase (*metB*) and cystathione β -lyase (*metC*). This branch uses cysteine and *O*-acetyl-homoserine to yield cystathione and then homocysteine in a two-step reaction. Deletion of *metB* in *M. tuberculosis* did not lead to auxotrophy (50), which suggests an alternative pathway. *M. tuberculosis* and other actinobacteria (65) also encode a cysteine-independent enzyme, *O*-acetylhomoserine sulfhydrylase encoded by *metY* (Rv3340), that catalyzes a sulfhydrylation reaction converting *O*-acetyl-homoserine and sulfide to homocysteine. Given the distance of CysA and CysH enzymes from the methionine biosynthesis pathway, it is unlikely that these strains are only methionine auxotrophs, and hence other metabolites can potentially lead to *in vivo* complementation of these mutants. We have recently shown that *M. tuberculosis metA*, a strain deficient in catalyzing the first reaction of methionine biosynthesis, could be chemically complemented by all intermediates of methionine biosynthesis (*O*-acetylhomoserine, homocysteine, methionine, or *S*-adenosylmethionine) *in vitro*, but no viable bacilli could be retrieved from immunocompetent and immunocompromised mice 3 weeks after infection or later (57). Hence, our study clearly showed that *M. tuberculosis* is unable to scavenge methionine or any of the pathway intermediates from mouse organs or human macrophages. Another interesting characteristic of the *metA* mutant was rapid killing and culture sterilization, which makes this pathway very attractive for drug discovery.

Histidine: A histidine auxotroph of *M. tuberculosis* was constructed by deleting *hisDC*, the first two genes of the *his* operon (66). This strain was attenuated in macrophages and rapidly killed when starved for histidine (66). However, the strain could survive prolonged periods in total starvation (water without any supplements) (66). The author concluded that histidine is not available to *M. tuberculosis* in THP-1 macrophages and that complete starvation of the histidine auxotroph might not reflect the *in vivo* situation because CFUs were clearly dropping in macrophages but not in water (66). Furthermore, it needs to be mentioned here that the *hisDC* mutant was not complemented, and hence the genotype-phenotype link is not yet confirmed.

Proline: A proline auxotroph in *M. tuberculosis* was created by deleting *proC* by homologous recombination and replacing the gene with a hygromycin cassette (66). This mutation was bactericidal in unsupplemented medium, and the mutant was attenuated in mice (66). Interestingly, CFU data showed that the mutant could still replicate in mouse organs, albeit at a very slow rate (66), which implies that proline can be scavenged *in vivo*. However, the mutation was not complemented, and colonies isolated from mice were not checked for suppressor mutations (66). It is conceivable that the *pruB*-encoded proline dehydrogenase (67, 68) is potentially capable of reversing the reaction and producing proline

in the absence of ProC. Future experiments will need to confirm *proC* and proline biosynthesis as viable drug targets.

Lysine: Construction of a lysine auxotrophic strain of *M. tuberculosis* was surprisingly difficult. Pavelka and Jacobs created *lysA* deletion mutants of *Mycobacterium smegmatis* and *M. bovis* BCG by allelic exchange using lysine-supplemented plates for rescue (69). However, no *lysA* mutants of *M. tuberculosis* could be isolated by this strategy until the lysine concentration in the medium was increased to 1 mg/ml and Tween 80 was added to the medium (69). The *lysA* mutants grew considerably slower than WT on solid media and in liquid media and always required Tween 80 for growth (69). Such dependence on detergent and a high concentration of supplement points to inefficient lysine uptake in this mutant. Whereas *M. smegmatis* efficiently transports lysine across the cell wall, *M. bovis* BCG and *M. tuberculosis* seem to lack a dedicated high-affinity lysine permease (M. Berney and G. M. Cook, unpublished results). Moreover, Pavelka and Jacob's results point to a difference in lysine uptake activity between *M. bovis* BCG and *M. tuberculosis*. The *M. tuberculosis lysA* auxotroph was strongly attenuated *in vivo* as it was rapidly cleared from mouse lungs after high-dose intravenous infections. Even when the same mice were injected with this strain three consecutive times, the lung burden dropped by 2 to 3 logs within 20 days, which strongly argues that lysine cannot be scavenged from the mouse organs. The attenuation described above made the *M. tuberculosis lysA* mutant attractive as a live attenuated vaccine candidate (53). The strain was made safer by adding a second auxotrophy (pantothenate) by knocking out *panCD* (70). This double mutant was then shown to be safe in guinea pigs as well as in non-human primates (71) and has since been approved for biosafety level II work by dozens of institutional biosafety committees from research organizations and universities (71).

Threonine: Covarrubias et al. (72) constructed a threonine auxotroph of *M. tuberculosis* by deleting *thrC*, the last enzyme in threonine biosynthesis. This mutant could not grow in the absence of threonine and grew slowly even in the presence of threonine. A systematic investigation of this mutant *in vitro* and *in vivo* has yet to be conducted to determine if threonine can be scavenged from the host or if threonine auxotrophy is lethal.

Leucine: Leucine auxotrophy in *M. tuberculosis* is one of the best-described amino acid auxotrophies in mycobacteriology (49). This stems from early studies showing that deletion of *leuD* yielded *M. tuberculosis* that was unable to establish an infection when delivered by aerosol to immunocompetent or immunocompromised mice and that the mutant was cleared quickly from mouse organs when given intravenously (49). Subsequently, a *leuD* strain was proposed as a live attenuated vaccine strain and was continuously improved to make it safer by knocking out two subunits in the leucine biosynthesis pathway (*leuC* and *leuD*) and by adding a second auxotrophy for pantothenate (*panCD*) (58, 73). Many labs now use this double auxotroph in biosafety level II laboratory conditions.

Isoleucine and valine: The biosynthetic pathways for the branched-chain amino acids isoleucine, valine, and leucine share several enzymes. Deletion of *ilvB1*, encoding the acetohydroxy-acid synthase, the key enzyme in branched-chain amino acid biosynthesis,

leads to multiple auxotrophies for isoleucine, valine, and leucine (74). Although several potential homologs are encoded in the *M. tuberculosis* genome (IlvB1, IlvB2, IlvG, IlvX), deletion of *ilvB1* led to complete loss of viability and killing in the absence of the three amino acids *in vitro* (74). Intravenous injection of this mutant into BALB/c mice showed that *M. tuberculosis ilvB1* is unable to proliferate in lungs or spleen. However, the strain was not cleared from the organs as has been observed for other auxotrophic *M. tuberculosis* strains (56). The authors concluded that there is potentially a homologous enzyme that is only activated *in vivo* and that can partially complement the loss of IlvB1 during mouse infection.

Tryptophan: The picture emerges that *M. tuberculosis* lacks the ability to take up most essential amino acids from the host. This is supported by the fact that no natural amino acid auxotrophs have been identified to date and that *M. tuberculosis* harbors the biosynthetic machinery for all 20 amino acids. The question remains of which immune mechanisms are responsible for amino acid deprivation in the infected host cells. Does the deprivation simply result from the fact that *M. tuberculosis* resides in a host-derived, nutrient-depleted vacuole, or are there other active amino acid depletion mechanisms at work? A recent publication on tryptophan auxotrophy helped to shed some light on this question (7). The first tryptophan auxotroph was isolated in 1999 by Parish et al. by deleting *trpD* from the *M. tuberculosis* H37Rv genome (48); the deletion was subsequently shown to be bactericidal *in vitro* in the absence of tryptophan supplementation, and the resulting mutant was deficient in growth in SCID mice and THP-1 macrophages (50, 66). Much later, by screening with “TraSH,” a method for mapping transposon insertion sites, several tryptophan biosynthetic genes associated with the bacterium’s ability to survive in MHC-II class knockout mice were identified (7). These mice are deficient in producing CD4 T cells, and it is well documented that CD4 T cells produce interferon (IFN), which in turn potently stimulates expression of IDO. IDO degrades tryptophan to kynurenine, thereby depleting tryptophan from macrophages and inhibiting the replication of various intracellular pathogens such as *Toxoplasma gondii* and *Chlamydia pneumoniae* (13–15).

The study by Zhang et al. was the first to suggest that tryptophan can be scavenged by *M. tuberculosis* from the host, albeit only when the host IDO degradative pathway is inhibited (7). Apart from arginine depletion by NOS and arginase (60) (see above), this is the only well-characterized immune mechanism that depletes an amino acid. It is interesting to note the difference in phenotype of a *trpD* mutant in the SCID mouse (no growth of *trpD*) compared to results in the MHC-II knockout mouse (growth of *trpD* and *trpE*). Both types of mice should be deficient in producing CD4 T cells and therefore IFN- γ . This discrepancy might argue that upon *M. tuberculosis* infection, SCID mice, which are deficient in mounting an adaptive immune response, still produce IDO. Indeed, it has been shown that IFN- γ is produced in SCID mice, possibly by natural killer cells (75–77). However, it is unclear if and why this mechanism would be absent in MHC-II knockout mice.

Glutamine: Glutamine synthetase (GS) is an integral part of central nitrogen metabolism in bacteria because it assimilates inorganic ammonium by condensation with glutamate to produce glutamine in an ATP-dependent manner. In *M. tuberculosis*, GS is encoded by

glnA1 (78, 79). Three other isoforms of GS are encoded in the *M. tuberculosis* genome (*glnA2*, *glnA3*, and *glnA4*), and all were shown to catalyze glutamine synthase activity *in vitro*, but only GlnA1 is abundantly expressed (78) and essential for bacterial homeostasis.

In addition to its role in bacterial nitrogen metabolism, *M. tuberculosis* GS plays an essential role in cell wall biosynthesis via the production of a poly-L-glutamate-glutamine, a cell wall component exclusively found in pathogenic mycobacteria (80). Interestingly, GS is found in high abundance extracellularly, possibly due to its high expression level and protein stability in the extracellular space (81). Deletion of *glnA1* in *M. tuberculosis* yielded a mutant with no detectable GS protein or GS activity and that was auxotrophic for L-glutamine (79). This glutamine auxotroph was rapidly killed *in vitro* in unsupplemented medium, attenuated for intracellular growth in human THP-1 macrophages, and no bacteria could be recovered from guinea pigs 10 weeks after infection. This argues that glutamine is not available for *M. tuberculosis* in guinea pig lungs. However, Beste and coworkers presented data showing glutamine to be taken up into WT *M. tuberculosis* cells from within macrophages (30). These discrepancies illustrate the knowledge gaps we still face in our understanding of *M. tuberculosis*-host metabolic cross-talk, and it remains to be tested if glutamine can be scavenged during latent infection or in immunocompromised animals. Nevertheless, *M. tuberculosis* GS is being actively pursued as a drug target, and several compounds have shown inhibition and *in vivo* efficacy. It is thought that the extracellular location of the bulk of the enzyme might obviate problems associated with the uptake of compounds across the notoriously impermeable mycobacterial cell wall (82).

Glutamate: Glutamic acid is a central player in nitrogen metabolism in *M. tuberculosis* (83). Emerging evidence in bacteriology suggests that glutamate, glutamine, and alpha-ketoglutarate are the three main metabolites of a metabolic feedback loop that integrates information from carbon and nitrogen metabolism and aids in the control of the carbon-nitrogen ratio in the cell (84). The standard medium for growing *M. tuberculosis*, 7H9, contains ample amounts of glutamate (3.4 mM), indicating a growth-enhancing role of this amino acid for this pathogen. It has been shown that *in vitro*, glutamate is a preferred nitrogen source, because *M. tuberculosis* grows faster on it than with ammonium (85, 86).

Creating a true glutamate auxotroph might be challenging because this amino acid is the substrate or product of many cellular reactions. Glutamine oxoglutarate aminotransferase (GOGAT) is a major glutamate-producing enzyme and is important in the nitrogen-sensing cycle that is regulated by GarA and PknG (87–89). Deletion of *gltBD*, the genes encoding the small and large subunit of GOGAT in *M. bovis* BCG, severely impaired growth of the mutant in ammonium-containing medium but did not completely abrogate it, indicating that other glutamate-producing processes can partially complement the loss of GOGAT function (90). Addition of glutamate, aspartate, and asparagine rescued the mutant to some degree, but only a combination of ammonium and high levels of glutamate brought growth of *gltBD* back to WT levels (90). Both aspartate and asparagine can be converted to glutamate by the bacterium.

The *gltBD* strain was able to grow in bone marrow-derived macrophages, indicating that its partial glutamate auxotrophy is complemented during infection (89). On the contrary, a BCG

mutant lacking the major glutamate catabolic enzyme glutamate dehydrogenase (Gdh) was severely attenuated for survival in RAW 264.7 macrophages and growth in bone marrow-derived macrophages (89). *M. bovis* BCG *gdh* was also unable to grow with cholesterol as the sole carbon source (89), indicating that glutamate anaplerosis (glutamate feeds into the TCA cycle via alpha-ketoglutarate) is important during growth on lipids and fatty acids. In fact, an *M. tuberculosis* strain (*pykA*) deficient for growth on fermentable carbon sources was recently shown to be unable to grow on fatty acids unless the medium was supplemented with glutamate (34). However, the *pykA* mutant was not attenuated in a mouse model, suggesting that metabolites such as glutamate or acetate can be scavenged from the host (34). An isotopologue labeling experiment in human THP-1 macrophages suggests *M. tuberculosis*'s ability to scavenge glutamate (30), but proof of glutamate utilization during animal infection is still missing. Constructing *gltBD* and *gdh* in *M. tuberculosis* and testing them in a mouse model could potentially shed some light on *M. tuberculosis*'s ability to scavenge glutamate from the host; however, ultimately, a true glutamate auxotroph has to be constructed to definitively answer this question, an endeavor that may entail the combination of multiple mutations in one strain.

Amino acids for which no auxotrophs have been constructed yet: Out of the 20 amino acids, auxotrophs for alanine, asparagine, aspartate, glycine, phenylalanine, serine, and tyrosine have, to our knowledge, not yet been isolated. Some of these metabolites (e.g., aspartate) are products of many enzymatic reactions, and therefore construction of auxotrophic strains can be challenging. Still, some of the biosynthetic pathways that produce these amino acids are worth looking at as potential drug targets even if multiple enzymes might have to be inhibited to achieve synthetic lethality.

Cofactor auxotrophies

Nicotinamide: *M. tuberculosis* produces nicotinamide adenine dinucleotide (NAD) by *de novo* biosynthesis but also encodes a functional salvage pathway (54, 91). This pathway allows the bacterium to use exogenous nicotinamide to complement NAD auxotrophy (54, 91). Two studies have investigated NAD auxotrophy in *M. tuberculosis* pathogenesis (54, 91), and both concluded that nicotinamide can be scavenged from the host, thereby allowing strains that are deficient in *de novo* biosynthesis of NAD to grow normally in mice and remain virulent. However, if the last two enzymes in NAD biosynthesis (NadD or NadE) are targeted, *M. tuberculosis* cannot be chemically rescued anymore, because these enzymes are also needed to produce NAD regardless of whether the precursors are made *de novo* or are salvaged (54, 91). *nadE* and *nadD* deletions are bactericidal, and conditional knockdowns cannot grow or survive during any stage of infection (92, 93). This makes NadD and NadE potential drug targets (93), yet the existence of human homologs could potentially complicate the finding of suitable inhibitors. To date, nicotinamide remains one of the few metabolites that have been convincingly shown to be scavenged from the host.

Pantothenate (vitamin B₅): Pantothenic acid is synthesized in bacteria, plants, and fungi, whereas it is a nutritional requirement in higher animals. This vitamin is a precursor of the essential cofactor coenzyme A (CoA). Sambandamurthy et al. (56) constructed a pantothenate auxotrophic strain of *M. tuberculosis* by deleting *panC* and *panD*, reasoning

that such a mutant should be severely impaired in global lipid biosynthesis. This mutant was attenuated in immunocompetent mice, showing a very slow reduction of CFUs in lungs (1 log/250 days) after high-dose intravenous infection. In SCID mice, which lack an adaptive immune response, the *panCD* mutant was able to replicate, albeit at a very slow rate. This is a very interesting observation and indicates that, in the absence of adaptive immunity, *M. tuberculosis* can potentially scavenge pantethine from the host. However, the possibility of a suppressor mutation was not ruled out.

Interestingly, recent work from our laboratory has shown that pantothenate auxotrophy is bacteriostatic for prolonged periods in unsupplemented standard medium, indicating that the consumption of this vitamin is very slow and/or that it can be efficiently recycled (57; M. Berney, unpublished results). Indeed, a *panK* conditional knockdown strain did not show significant attenuation in a mouse infection model even in the absence of inducer, which means that inactivation of pantothenate biosynthesis is not immediately bactericidal and that PanK is not a good drug target (94). Whether the CoA biosynthetic pathway harbors any viable drug targets remains to be assessed. Nevertheless, the *panCD* mutant showed great potential as a vaccine strain because it gave better protection in mice and was safer than BCG (56). Subsequently, a *panCD* deletion was used to create several *M. tuberculosis* strains (*panCD lysA*, *panCD leuD*, *panCD RDI*) that were tested in guinea pigs and non-human primates for their vaccine potential (56, 70, 71, 73, 95). In all these experiments, the auxotrophic strains were safer than BCG and showed protection similar to the current BCG vaccine. From a nutritional point of view, it could be interesting to pursue the slow growth phenotype of *panCD* in SCID mice because it indicates the existence of an immune mechanism that restricts *M. tuberculosis* from accessing dietary pantethine from the host.

Pyridoxamine (vitamin B₆): *M. tuberculosis* synthesizes pyridoxal 5-phosphate (PLP), the bioactive form of vitamin B₆, by a bifunctional enzyme complex called PLP synthase, a class I glutamine amidotransferase composed of the synthase domain Pdx1 and the glutaminase domain Pdx2. In mycobacteria, PLP is predicted to be the cofactor of 58 proteins, many of which are predicted to be essential. Pyridox-amine is a vitamere of vitamin B₆ and a supplement in the standard medium of *M. tuberculosis*. Dick and coworkers constructed a *pdx1* knockout strain in *M. tuberculosis* that is a vitamin B₆ auxotroph (52). Chemical complementation was reached at the relatively low concentration of 5 μ M pyridoxine (52). Vitamin B₆ auxotrophy was bactericidal in unsupplemented medium under a variety of conditions (exponential growth, stationary phase, hypoxia), albeit at a slow rate compared to other auxotrophs (52). *M. tuberculosis pdx1* was unable to establish an infection in immunocompetent mice after aerosol infection, and the mutant was cleared from the lungs after 30 days (52). To our knowledge, the same mutant was not tested in immunocompromised mice; hence, it is unknown if vitamin B₆ is actively withheld from the pathogen by the immune system.

Pdx1 looks like a promising drug target, but an assessment of its essentiality during latent infection (such as by conditional knockdown) and in immunocompromised mice has yet to be conducted. To this end, it is interesting to note that *pdx1* was recently deleted in the vaccine strain *M. bovis* BCG *ureC::hly* to increase safety (96). In mice that received

vitamin B₆ supplements, BCG *ureC::hly pdx1* exhibited prolonged survival in the draining lymph nodes, but not in spleens (96), which argues that pyridoxine can be scavenged from the host if it is available in high enough concentrations. The authors further concluded that the improved survival of the auxotrophic BCG *ureC::hly pdx1* strain, due to administration of vitamin B₆, supported the generation of memory T cells, which persisted after clearance of the vaccine strain (96).

Biotin (vitamin B₇): Biotin is an essential cofactor for enzymes in metabolic pathways such as fatty acid biosynthesis, anaplerosis, and amino acid metabolism (97). Biotin auxotrophy was the first auxotrophy in *M. tuberculosis* that was shown to be bactericidal during latent infection in mice (51). This was an important milestone because it showed that, even in this putatively more quiescent stage of pathogenesis, auxotrophy could lead to death. Woong Park and coworkers constructed a BioA conditional knockdown strain with a tetracycline-responsive genetic switch to turn on or off the *bioA*-encoded 7,8-diaminopelargonic acid synthase (BioA) (51). This strain allowed BioA expression to be controlled during different stages of infection and demonstrated that *M. tuberculosis* is not able to scavenge biotin from the mouse at any stage of infection (51). Such information is particularly useful for drug target discovery, because new anti-TB drugs should kill both actively growing bacteria and nongrowing, persistent bacteria. This same group and others have already developed some promising lead compounds that inhibit BioA and that are efficacious against *M. tuberculosis* whole cells (98). Using conditional knockdowns, these investigators have developed a new drug screen called the “target-based whole cell screen.” The advantage of using a conditional knockdown is that the bacterium can be sensitized to a drug by partially depleting the target enzyme, thereby detecting potential lead scaffolds that might be ignored in traditional, whole-cell drug screens due to their high MICs (98–100).

Folate (vitamin B₉): Folates are essential cofactors in many one-carbon transfer reactions and are required for the production of purines, pyrimidines, and certain amino acids. The essentiality of reduced folates in cellular metabolism made folate biosynthesis a clinically important target of drugs to treat cancer as well as bacterial (including *M. tuberculosis*), fungal, and parasitic infections. Inhibitors of folate biosynthesis have been studied as TB chemotherapeutics since the discovery in the 1940s that para-aminosalicylic acid (PAS) has antitubercular activity (101–104). Although it was initially believed that the main mechanism of action of PAS is via inhibition of dihydropteroate synthase (DHPS), it was shown recently that PAS acts as a prodrug by poisoning folate-dependent pathways (103). Among the enzymes involved in folate biosynthesis, dihydrofolate reductase has been the target of numerous drug screens (101, 105, 106). Some of these drugs, such as sulfamethoxazole or dapsone, have some efficacy *in vivo*, which argues that folate or its precursors cannot be scavenged from the host in sufficient amounts to relieve the metabolic block. However, in the case of PAS, the exact mode of action is still unknown, and it is suggested that multiple targets are affected by this drug. A genetic approach with auxotrophic strains or conditional knockdowns could shed some light on the availability of folate intermediates in host tissues.

Cobalamin (vitamin B₁₂): To date, it is still unclear if *M. tuberculosis* relies on vitamin B₁₂ uptake during pathogenesis. *M. tuberculosis* encodes genes for *de novo* vitamin B₁₂ biosynthesis (107), but it has been suggested that the production of this cofactor is hampered or even abrogated due to the absence and mutation of certain genes (108, 109). Based on bioinformatics analyses, it is possible that *M. tuberculosis* produces B₁₂, but a growth condition has yet to be identified where endogenous B₁₂ production is induced, and Gopinath and coworkers argue that this induction might only happen *in vivo* (108). Three unrelated B₁₂-dependent enzymes and one B₁₂ transporter have been identified in *M. tuberculosis* (108, 109). Investigations into their functions helped in understanding several aspects of B₁₂ metabolism in this pathogen.

Growth of *M. tuberculosis* on odd-chain fatty acids or cholesterol as single carbon sources was shown to depend on the detoxification of propionate catabolite accumulation (110–112). This is an important finding because *M. tuberculosis* is believed to rely primarily on a lipid-rich diet *in vivo*, which requires β -oxidation that produces propionyl-CoA as well as acetyl-CoA. Accumulation of toxic propionyl-CoA can be prevented by a functioning methylmalonyl-CoA pathway or methylcitrate cycle (111). The methylmalonyl-CoA pathway is B₁₂ dependent, due to methylmalonyl-CoA mutase (MCM) (111), whereas the methylcitrate cycle is B₁₂ independent. Although MCM is not essential for virulence, it is intriguing that *M. tuberculosis* keeps a B₁₂-dependent and a B₁₂-independent mechanism to detoxify propionyl-CoA. This redundancy is also observed in the last step of methionine biosynthesis, where MetH encodes a vitamin B₁₂-dependent methionine synthase and MetE encodes a B₁₂-independent enzyme (113).

Being able to quickly respond to changes in B₁₂ availability might highlight the importance of methionine biosynthesis and detoxification of propionyl-CoA for the bacterium. We have shown that inactivation of methionine biosynthesis is extremely lethal to *M. tuberculosis* (57). Controlling methionine and *S*-adenosylmethionine biosynthesis with such a metabolic switch could argue that B₁₂ is a metabolic checkpoint during infection. However, some clinical isolates, for example, *M. tuberculosis* CDC1551, carry an inactive (through truncation or other mutation) version of MetH (113). This strain is susceptible to B₁₂ (113), because it inhibits transcription of MetE and, with it, the biosynthesis of the essential amino acid methionine and the cofactor *S*-adenosylmethionine (57). Since this strain is still virulent, B₁₂ obviously does not play a role in its pathogenesis. Still, *M. tuberculosis* encodes a dedicated B₁₂ transporter (114), which enables the pathogen to take advantage of B₁₂ availability when it might occur.

Other auxotrophies: purine—Two purine auxotrophic strains were constructed and characterized in *M. tuberculosis* and *M. bovis* BCG with the goal to create new live vaccines (115). Deletion of the gene *purC* rendered both strains unable to grow in the absence of hypoxanthine (115). In macrophages, both the *M. tuberculosis* and BCG auxotrophs could not proliferate, but there was a difference in survival (115). Whereas the *M. tuberculosis purC* strain persisted, the *purC* mutant of BCG was killed gradually (115). After intravenous injection into BALB/c mice, the *M. tuberculosis purC* mutant persisted in lungs, spleens, and livers for about 20 days, after which the CFU burden in all organs started to drop and became undetectable after 60 days (115). The temporary persistence of the *M.*

tuberculosis purC mutant in BALB/c mice is most likely due to residual hypoxanthine/purine after growth in supplemented medium before injection. Temporary persistence of an *M. tuberculosis* auxotroph *in vivo* might be a useful trait for a live vaccine because it is thought that persistence of vaccine strains through limited replication *in vivo* generates better protective immunity against *M. tuberculosis* (116). The *purC* mutant was also tested in a guinea pig model to evaluate its protective efficacy against *M. tuberculosis* infection. Protection after subcutaneous vaccination with 10^7 CFU/ml *M. tuberculosis purC* was similar to BCG in the lungs, but the *M. tuberculosis* mutant was less protective than BCG in the spleen. Taken together, these data lead to the conclusion that *M. tuberculosis* cannot scavenge purine or hypoxanthine from the host in amounts sufficient for normal growth.

CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the current literature, *M. tuberculosis* has the capacity to take up most amino acids and cofactors *in vitro*. However, this ability to scavenge essential building blocks does not translate to the *in vivo* situation in the presence of intact innate and adaptive immunity. For example, *M. tuberculosis* is predicted to have up to five putative arginine transporters (117), yet an arginine auxotroph is unable to survive in a mouse infection model (55). Moreover, to our knowledge, only one example is known to date where absence of adaptive immunity aids the proliferation of an *M. tuberculosis* auxotroph (tryptophan) (7). This does not necessarily mean that *M. tuberculosis* lacks the ability to take up amino acids from the host, but it argues that not enough metabolites can be scavenged to chemically complement the auxotrophies. It is conceivable that *M. tuberculosis* feeds on a complex host diet whenever it is available but that this happens at concentrations that are orders of magnitude smaller than what is needed to offset an auxotrophy. Nutritional immunity is likely responsible for this phenomenon. In other intracellular pathogens that are naturally amino acid dependent, such as *L. pneumophila* (16), *F. tularensis* (17), and *L. monocytogenes* (18), auxotrophy always comes with a sophisticated virulence mechanism allowing the pathogen to circumvent nutritional immunity by manipulating the host to provide large amounts of the needed growth factor. The human pathogens evolved to access host nutrients by stimulating host protein degradation, manipulation of autophagy, or degradation of complex metabolites such as glutathione. Such mechanisms have been described as nutritional virulence (19) and are direct reactions to nutritional immunity. These adaptations might also have evolutionary consequences because the loss of genes required for synthesis of an amino acid results in partial dependency on the host, and the bacteria will coevolve with the host (118).

Mycobacterium leprae, a close relative of *M. tuberculosis*, is an extreme example of genome reduction (119). Its metabolism is so much adapted to the host that it cannot be cultured axenically. Indeed, genetic links between new metabolic capacities and virulence factors illustrate that metabolic pathways are acquired as part of a pathogen's evolution toward colonizing new niches with new food sources (120). Such mechanisms are, to date, unknown in *M. tuberculosis*, and it will be interesting to see if any of the five type VII secretion systems present in the *M. tuberculosis* genome (ESX-1 to ESX-5) (121) are involved in nutrient acquisition. To this end, the ESX-3 gene products were proposed to be involved in iron acquisition (122), and ESX-1 is involved in phagosomal escape, potentially giving access to cytosolic nutrients. The phagosomal milieu is generally inherently nonpermissive

for bacterial growth in comparison to the cytosol (123). For example, *L. monocytogenes*, which is naturally auxotrophic for several amino acids and vitamins (124), replicates within the cytoplasm, but hemolysin-negative mutants, which are unable to escape from the phagosome, do not grow (125). However, although the cytosol is believed to be amino acid replete, it is questionable if the nutrient abundance is enough to allow considerable replication of pathogens. According to Abu Kwaik and Bumann (126), extensive proliferation based on host-derived amino acids and energy sources is only possible if nutrients are replenished through active transport into the phagocytes.

The amino acid and cofactor independence of *M. tuberculosis* allows this bacterium to dwell on a minimal diet of carbon, nitrogen, and trace elements in the host. Intracellular pathogens compete with the host cell for the same nutrient pools, and hence, a strategy of using a minimal amount of host metabolites means not leaving a trail, thereby potentially gaining more time for proliferation as an unrecognized intruder. This feature might also be connected to *M. tuberculosis*'s ultraslow growth rate. Slow growth allows *M. tuberculosis* to preferentially use lipids as carbon and energy sources while making the whole set of essential amino acids by itself. It is intriguing to note that most intracellular pathogens that are natural auxotrophs grow considerably faster in the host. For example, *F. tularensis* divides every 3 hours in human alveolar macrophages (129), *L. pneumophila* every 2 hours (130), and *L. mono-cytogenes* doubles in less than an hour (131); in contrast, the maximum specific growth rate of *M. tuberculosis* in the same type of macrophage is 24 hours. Faster growth means higher energetic needs, and amino acid and cofactor biosyntheses are energetically expensive processes, so uptake of the finished building blocks can be a huge energetic advantage. This is reflected in the slower growth rate of bacteria when growing on minimal medium compared to a rich medium that contains many building blocks. For example, the doubling time of *E. coli* triples from 20 minutes in Luria broth to 1 hour on minimal medium (127). Fast proliferation, though, also means the need for fast replenishment of those nutrients, which many intracellular pathogens achieve by manipulating the host to provide more of the precious food. In contrast, it seems as if the intracellular lifestyle of *M. tuberculosis* is more focused on remaining as metabolically quiet as possible, thereby subverting many potential assaults by the immune system. Collectively, the literature reviewed here suggests that nutritional independence from the host is an important virulence mechanism of *M. tuberculosis* that holds great promise for new drug target discovery.

Several new studies show that amino acid and cofactor auxotrophy in *M. tuberculosis* can be bactericidal and lead to rapid killing *in vitro* and *in vivo* (7, 57, 92), which leads to the conclusion that unbalanced growth/metabolism is a bactericidal event that should be considered for drug target discovery. Intriguingly, Parish (66) showed that killing of *M. tuberculosis* auxotrophs was very slow in a general starvation medium such as phosphate-buffered saline but more rapid in unsupplemented medium (medium that only lacks a particular nutrient). This phenomenon is called "unbalanced growth" and was first discussed in a paper by Cohen and Barner published in 1954 on thymine-less death in *E. coli* (128). The authors state, "The induction of unbalanced growth in this case [i.e., thymine auxotroph in thymine-depleted medium] leads to death; the inhibition of all growth, as a result of the

omission of many metabolites, permits survival.” In fact, most, if not all, antibiotics on the market kill replicating cells much more efficiently than nonreplicating cells.

These considerations also lead to the question of whether complete starvation (e.g., using water or phosphate-buffered saline) is a good model of the *in vivo* environment of *M. tuberculosis*. Mouse experiments with several amino acid auxotrophs show clearance of *M. tuberculosis* from lungs, spleens, and liver whether infected by aerosol or intravenously (49, 53). Hence, the current data clearly argue that the *in vivo* nutritional environment is more akin to unsupplemented medium (possibly due to the constant availability of a carbon source) and is not perceived by the pathogen as complete starvation. However, this still has to be systematically investigated because the lethality of inactivating amino acid biosynthesis has not been shown during the latent phase of infection. The natural route of *M. tuberculosis* infection is via aerosol into the lungs, and most auxotrophs examined so far were unable to proliferate in the lung and therefore could not establish a latent infection. With the latest genetic tools for mycobacteria, conditional knockdowns, we can now examine auxotrophies during different stages of infection, e.g., active and latent, as has been nicely shown for three auxotrophs so far: a biotin auxotroph, a nicotinamide auxotroph, and a methionine auxotroph (M. Berney, unpublished data). Using this technology in a systematic fashion will allow us to learn about new mechanisms of nutritional immunity in TB pathogenesis.

The ultimate goal of this research must be to defeat the TB disease. *M. tuberculosis*'s strong dependence on its own amino acid and coenzyme biosynthesis machinery allows it to be independent from the host, stay metabolically flexible, and keep a low profile during infection. At the same time, the absence of virulence mechanisms to acquire large amounts of host amino acids and cofactors makes the biosynthetic machinery an attractive vulnerability for drug targeting. Inhibition of biosynthetic pathways holds great promise for discovering new drug targets because it seems that *M. tuberculosis* finds itself in an amino acid- and cofactor-deprived environment in the host. However, not all biosynthetic pathways are suitable for targeting. For example, some amino acids, such as alanine and glutamate, can be produced by multiple enzymes, which makes the search for suitable inhibitors more difficult. Much work is ahead of us to identify the right pathways and the right enzymes that lead to rapid killing and that, most importantly, are going to be amenable to drug targeting.

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