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Feast or famine: the host–pathogen battle over amino acids

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Summary

Intracellular bacterial pathogens often rely on their hosts for essential nutrients. Host cells, in turn, attempt to limit nutrient availability, using starvation as a mechanism of innate immunity. Here we discuss both host mechanisms of amino acid starvation and the diverse adaptations of pathogens to their nutrient-deprived environments. These processes provide both key insights into immune subversion and new targets for drug development.

Introduction

Successful bacterial pathogens exploit their hosts to support their own survival. Pathogens rely on their hosts for nutrients necessary for survival, injuring the hosts in the process. For their part, the hosts are hardly gracious. In fact, the ability to keep would-be pathogens away from the nutrients turns out to be a key part of host defence. This interplay exists for all essential nutrients, including carbon, nitrogen and transitional metals (Eisenreich *et al.,* 2010; Skaar, 2010; Rohmer *et al,* 2011; Hood and Skaar, 2012).

For many potential pathogens, amino acids are critical nutrients. While many organisms can synthesize their own amino acids, others must scavenge them in order to make proteins. Additionally, many bacterial pathogens are able to use amino acids as a carbon source, some even as their principle carbon source (Molofsky and Swanson, 2004; Wieland *et al,* 2005; Eylert *et al,* 2010; Venugopal *et al.,* 2011). Accordingly, mammalian hosts have evolved mechanisms to starve bacteria of amino acids. And, to counter this, bacteria have a diversity of means to respond to this stress.

Here we will review the evidence for and mechanisms of host-mediated amino acid starvation and the bacterial response to amino acid depletion. We have chosen three intracellular pathogens to illustrate the diversity of bacterial responses. The first, *Chlamydia trachomatis,* is a natural auxotroph for many amino acids and responds to amino acid starvation primarily by differentiating to a viable but non-replicating form. The second, *Mycobacterium tuberculosis,* has evolved to become independent of host amino acid availability and constitutively synthesizes its own amino acids. The third, *Legionella pneumophila*, is able to extract amino acids from an otherwise stingy host by exploiting host machinery – including amino acid transporters and the host protea-some – to make amino acids available in the pathogen's intracellular niche.

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Host cells are able to starve intracellular pathogens of amino acids

The evidence

The exact intracellular niches of bacterial pathogens are difficult to isolate; thus their metabolite contents are difficult to measure directly. Pathogen adaptation to the intracellular environment, however, suggests that host cells are able to limit amino acid availability. The most compelling fact is that amino acid auxotroph strains of many bacterial pathogens are often attenuated for intracellular growth and during host infection. For example, *M. tuberculosis* requires the biosynthesis pathways for at least four amino acids to survive during a model infection of mice and within isolated mouse and human macrophages. Leucine, proline and lysine auxotrophs are attenuated *in vivo,* the latter by so much that they have been tested as live-attenuated vaccine candidates (Hondalus *et al,* 2000; Smith *et al.,* 2001; Pavelka *et al.,* 2003b). Additionally, multiple groups have shown the requirement of several tryptophan biosynthesis enzymes for survival in mice and macrophages (Smith *et al.,* 2001; Parish, 2003). *Salmonella* auxotrophs for aromatic amino acids, histidine and methionine have also been shown to be attenuated *in vivo* (Hoiseth and Stocker, 1981; Fields *et al.,* 1986; O'Callaghan *et al.,* 1988).

Measuring the pathogen's transcriptional response to the intracellular niche paints the same picture of amino acid starvation. Since pathogens often regulate gene expression in response to environmental signals, expression profiles can serve as bioprobes of the conditions they encounter. Many intracellular pathogens upregulate amino acid biosynthesis genes during infection (Chatterji and Ojha, 2001). This is true for bacterial pathogens as well as fungal pathogens like *Candida albicans* (Rubin-Bejerano et al., 2003). Many fungal species encode a kinase, Gcn4, that senses exogenous amino acid availability. Upon infection, Gcn4 is activated and coordinates *Candida* amino acid biosynthesis (Tripathi *et al.*, 2002). Additionally, the stringent response, a conserved bacterial signalling pathway that senses amino acid starvation, among other stresses, is often required for intracellular growth (Magnusson *et al.*, 2005; Potrykus and Cashel, 2008). RelA senses amino acid starvation and synthesizes ppGpp, which induces necessary adaptive changes, including transcriptional upregulation of amino acid biosynthesis genes (Traxler *et al.,* 2008). In many intracellular pathogens, including *M. tuberculosis* and *Salmonella,* RelA is upregulated and specifically required for intracellular growth (Primm *et al.,* 2000; Pizarro-Cerdá and Tedin, 2004; Song *et al.,* 2004; Thompson *et al.,* 2006).

The mechanisms

While there is much evidence that the host can create an amino acid depleted niche, the mechanisms for producing this starved niche are not well characterized. One major clue, however, comes from the autophagic response to intracellular pathogens. Autophagy is an important cellular process through which eukaryotic cells respond to a variety of stresses, including metabolite starvation and damaged organelles (Levine, 2005; Deretic, 2009; Deretic and Levine, 2009). Bacterial invasion also induces an autophagic response, called xenophagy, wherein invading pathogens are delivered to autophagosomes to be broken down (Levine, 2005; Deretic, 2009; Deretic and Levine, 2009). This process of xenophagy is required for optimal suppression of the growth of multiple intracellular pathogens, including

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Listeria, Shigella and *Mycobacterium* species (Gutierrez *et al.,* 2004; Singh *et al.,* 2006; 2010; Suzuki *et al.,* 2007; Zhao *et al.,* 2008; Yuk *et al.,* 2009).

Nutritional signals play a key regulatory role in autophagy. In particular, autophagy is inhibited when the cytosol contains high concentrations of amino acids. Tattoli and colleagues found that intracellular amino acid levels must be low in order for xenophagy to be fully induced (Sancak *et al.,* 2008; 2010). They found that the activity of mTOR, a checkpoint kinase that links nutrient sensing to metabolic activity, decreased upon infection with the intracellular pathogens *Salmonella* and *Shigella* (Tattoli *et al.,* 2012b). This indicated a possible amino acid starvation, as mTOR activity decreases when amino acid levels are low. Moreover, cytosolic levels of isoleucine and leucine fell within an hour after infection (Tattoli *et al.,* 2012b). Interestingly, *Shigella-induced* amino acid starvation lasted at least 4 h, while *Salmonella* induced a more transient 1 h starvation. The authors hypothesized that membrane damage might be responsible for amino acid starvation as the temporal pattern of such damage (transient for *Salmonella*; lasting for *Shigella*) matched the loss of amino acids. In fact, even aseptic membrane damage, induced by digitonin, could induce amino acid starvation. While this does not rule out alternative mechanisms, this study strongly supports the possibility that bacteria induce amino acid starvation and presents compelling evidence that membrane damage could play a major role (Tattoli *et al.,* 2012a,b). Interestingly, *Salmonella* could reverse this amino acid starvation, which activated mTOR and inhibited autophagy, ultimately restoring bacterial growth. When the authors overrode the starvation-induced autophagy block, Salmonella growth was restored (Tattoli *et al.,* 2012b).

In addition to general amino acid depletion, host cells can also specifically deplete intracellular tryptophan during pathogen invasion (Hayashi *et al.,* 2001; Silva *et al.,* 2002). Tryptophan depletion as an anti-bacterial mechanism was first described in *Chlamydia*-infected cells (Byrne *et al.,* 1986; Murray *et al.,* 1989; Beatty *et al.,* 1994). The addition of interferon-gamma (IFN-γ) to infected cells almost completely abolishes intracellular chlamydial growth. Stunningly, simply adding tryptophan reverses the anti-chlamydial effect of IFN-γ (Byrne *et al.,* 1986; Beatty *et al.,* 1994; Leonhardt *et al.,* 2007). IFN-γ activates indoleamine-2,3-dioxygenase (IDO), the enzyme responsible for the first step in kynurenine synthesis from tryptophan (Leonhardt *et al.,* 2007; Zelante *et al.,* 2009). When strongly induced – as in the case of IFN-γ activation – IDO can deplete about 95% of intracellular tryptophan (Fujigaki, 2002). Thus, IDO is required for limiting the growth of tryptophan-requiring intracellular pathogens, such as some *Chlamydia* species, *Leishmania donovani, Toxoplasma gondii,* as well as lab-generated strains tryptophan-auxotrophic bacteria (Daubener *et al.,* 2001; Fujigaki, 2002; Leonhardt *et al.,* 2007; Ibana *et al.,* 2011a,b).

It is worth noting, however, that the effect of IDO on pathogen growth is multi-faceted (Medzhitov *et al.,* 2011). The first appreciated role for IDO in pathogen defence was intracellular tryptophan depletion. Recently, the role of its enzymatic products, kynurenines, has also been characterized. Kynurenines are potent negative regulators of inflammation and T cell activity (Munn *et al.,* 2005; Zelante *et al.,* 2009; Favre *et al.,* 2010; Medzhitov *et al.,* 2011). So while the role of IDO on intracellular tryptophan is clear, its pleiotropic effects in pathogen defence have been more difficult to pin down (Blumenthal *et al.,* 2012).

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Nevertheless, IDO represents a mechanism by which an infected host can drastically reduce the amounts of an often-essential amino acid available to pathogen.

Pathogen responses to host AA starvation

Many pathogens are able to synthesize the full set of amino acids. It would be reasonable to think, given how common amino acid depleted environments are, that amino acid biosynthetic pathways would be a requirement for being an intracellular human pathogen. However, many known successful human pathogens are auxotrophic for some amino acids. How can this be? Some bacteria are simply able to escape by altering their metabolic requirements. For example, they can differentiate into non-replicating, less metabolically demanding cells. Other organisms are able to avoid amino acid starvation altogether by manipulating host amino acid uptake and production systems to increase available amino acids in the pathogen's intracellular niche. We will review three model pathogens that fit these three moulds: (i) *Chlamydia,* which employs growth arrest and differentiation, (ii) *M. tuberculosis,* which exemplifies amino acid self-sufficiency, and (iii) *L. pneumophila,* which exploits host machinery to extract amino acids from the host cell.

Chlamydia: *hiding out and holding out for a better day – growth arrest and differentiation*

Chlamydia trachomatis causes a variety of human diseases, including the leading cause of infectious blindness and genital tract infections that can have long-term severe consequences for infected individuals. All members of the *Chlamydia* family are intracellular pathogens that share two distinct developmental stages. Elementary bodies (EBs) initiate the intracellular infection through endocytic uptake. In optimal growth conditions, the metabolically inert EBs then differentiate into metabolically active reticulate bodies (RBs), which then replicate and grow within the bacterial vacuole. Eventually RBs develop back into EBs, which upon release, go on to infect other cells.

When stimulated with IFN-γ, the host cell imposes a much greater stress upon the bacteria. *Chlamydia* responds to this stress by morphing into a third, aberrant form (Beatty *et al.,* 1994; Leonhardt *et al.,* 2007). The aberrant form cells are similar to reticulate bodies in morphology, but are unable to divide or differentiate back to EBs. In some studies, adding tryptophan restores growth of these aberrant RBs, demonstrating that persistence is driven by tryptophan starvation (Byrne *et al.,* 1986; Beatty *et al.,* 1994; Leonhardt *et al.,* 2007; Ibana *et al.,* 2011a). Differentiation into this aberrant RB form allows the pathogen to hide from its tryptophan-depleted environment until tryptophan availability resumes (Fig. 1). Isoleucine also seems to be limited during infection, and *Chlamydia* growth in one intracellular infection model could be restored upon the addition of isoleucine (Hatch, 1975). Interestingly, *Chlamydia* does not encode the classical bacterial stringent response, which enables many bacteria to sense amino acid starvation and enter into persistent states (Ouellette *et al.,* 2006). Thus, *Chlamydia* must utilize a unique mechanism to sense tryptophan depletion and differentiate into these aberrant forms.

Early investigations on the role of tryptophan and IDO in driving aberrant form differentiation yielded mixed results. While some groups found tryptophan supplementation alone could reverse the effects of IFN-γ, others found no effect (Murray *et al.,* 1989). When

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the *C. trachomatis* genome was sequenced – and especially when multiple clinical strains were sequenced – a major clue emerged. As expected, *Chlamydia* lacks the full suite of tryptophan biosynthetic enzymes, and thus cannot synthesize tryptophan from chorismate or most other biosynthetic precursors (Stephens, 1998). It does, however, encode tryptophan synthase (TrpAB), a more limited enzyme that allows the organism to synthesize tryptophan from indole. In addition *Chlamydia* encodes TrpR, a tryptophan- dependent aporepressor, which enables the pathogen to react specifically to environmental tryptophan availability (Stephens, 1998; Belland *et al.,* 2003; Wood *et al.,* 2003). Interestingly, *C. trachomatis* strains isolated from ocular infections and synovial tissue contain frameshift mutations in *trpAB,* while strains from genital infections do not (Fehlner-Gardiner *et al,* 2002; Caldwell *et al,* 2003; Gerard *et al.,* 2010). Within strains isolated from sexually transmitted infections, there are three *trpAB* variants; while the effect of each *trpAB* variant on tryptophan synthesis is unknown, identity of the *trpAB* allele correlates perfectly with IFN-γ susceptibility *in vitro* (Morrison, 2000; Fehlner-Gardiner *et al,* 2002; Caldwell *et al.,* 2003; McClarty *et al.,* 2007). So while all *C. trachomatis* contain the *trpAB* gene, not all strains are able to make tryptophan, and the ability to make tryptophan appears to track with STI virulence.

Is the *C. trachomatis* response to tryptophan starvation differentiation to a non-growing cell type or regulated tryptophan biosynthesis? It appears that tryptophan biosynthesis is possible, but under a limited number of circumstances. First, the strain has to encode a functional enzyme. Second, it has to have a source of indole, which the host does not make. Some investigators have noticed that many functional TrpAB-containing strains were isolated from patients with bacterial vaginosis, and thus hypothesize that the vaginal flora might be a source of indole (McClarty *et al.,* 2007). So some *C. trachomatis* strains can synthesize tryptophan in certain environments, increasing their resistance to IFN-γ mediated immunity (Belland *et al.,* 2003). Where tryptophan biosynthesis is not available, *Chlamydia* use their ability to enter into a persistent form that enables it to survive when all sources of tryptophan are lost.

M. tuberculosis: *energy independence – making its own amino acids in an unreliable environment*

Whereas *Chlamydia* tryptophan biosynthesis is limited in scope, *M. tuberculosis* seems to leave nothing up to chance. It contains the entire biosynthetic toolset for all 20 amino acids (Cole *et al.,* 1998). During infection of both mice and macrophages, it appears that *M. tuberculosis* constitutively expresses amino acid biosynthesis genes (Schnappinger *et al.,* 2003; Talaat *et al.,* 2004; 2007; Rohde *et al.,* 2012). Even when grown *in vitro,* where tryptophan is freely available, the tryptophan biosynthetic locus continues to be expressed (Y. Zhang and E. Rubin, unpubl. data). Thus, *M. tuberculosis* seems to choose to independently synthesize amino acids regardless of environmental availability (Fig. 1). *M. tuberculosis* does the same with energetic sources as well – unlike *Escherichia coli, M. tuberculosis* will continue to metabolize energy- poor carbon sources even in the presence of more energy efficient carbon sources (de Carvalho *et al.,* 2010).

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The *M. tuberculosis* intracellular niche is similar to *Chlamydia's. M. tuberculosis* is taken up by phagocytosis and enters into a vacuole that escapes lysosome fusion. As with *Chlamydia,* there is strong evidence that IFN-γ activation of infected macrophages plays a major role in controlling *M. tuberculosis* growth (Flynn *et al.,* 1993; Fabri *et al.,* 2011). Unlike *Chlamydia, M. tuberculosis* is always able to make tryptophan, which is essential for its survival of IDO activation by IFN-γ. Its ability to make other amino acids is also crucial for virulence, as lysine, proline and leucine auxotrophs also fail to grow normally in macrophages and are severely attenuated in mice (Hondalus *et al.,* 2000; Smith *et al.,* 2001; Parish, 2003; Pavelka *et al.,* 2003a). Perhaps because amino acid biosynthesis is constitutive in *M. tuberculosis*, amino acid biosynthesis has not been seen as a virulence factor. However, these processes are clearly essential and, in fact, could serve as the basis for drug design.

If amino acid production is constitutive, is there any role for amino acid sensing during infection? *M. tuberculosis* does have a stringent response, and it encodes a RelA homologue, which in *E. coli* and other organisms responds to amino acid starvation (Primm *et al.,* 2000). ReIA knockouts are attenuated for growth in mice and guinea pigs (Primm *et al.,* 2000; Dahl *et al.,* 2003; Klinkenberg *et al.,* 2010). It is likely that RelA can respond to amino acid levels – this is true in a related species, *Mycobacterium smegmatis* – but neither RelA sensing of amino acid starvation nor RelA-dependent co-ordination of amino acid synthesis is well documented in *M. tuberculosis* (Dahl *et al.,* 2005). The stringent response signals through ppGpp, which in *E. coli* requires DksA to be fully active (Paul *et al.,* 2004; Magnusson *et al.,* 2005). *M. tuberculosis* encodes CarD, an alternative DksA, which is also required for the stringent response to starvation, and mutants are slightly attenuated in mice (Connolly and Cox, 2009). However, CarD is essential in rich media, suggesting a role outside of the stringent response that might explain its requirement for growth *in vivo* (Connolly and Cox, 2009; Stallings *et al,* 2009). Notably, RelA mutants are far less attenuated than amino acid auxo-trophs (Primm *et al.,* 2000). It is most likely that the stringent response in *M. tuberculosis* is primarily responsible for sensing and responding to other forms of starvation or immune-mediated stress. Thus, *M. tuberculosis* is an example of a pathogen that has decoupled amino acid metabolism from host availability. It persists in making its own amino acids, making it resistant to host mechanisms of amino acid starvation.

Legionella: *host exploitation – extracting amino acids from a stingy host*

Not all bacteria are as energy independent as *M. tuberculosis*. The host, after all, is a tremendous resource for essential nutrients if the pathogen can manage access (Rohmer *et al.,* 2011). In this respect, *L. pneumophila* has been quite successful. Like *M. tuberculosis* and *Chlamydia, L. pneumophila* replicates in an intracellular vacuole, the *Legionella-containing* vacuole (LCV). The LCV avoids lysosomal fusion and further develops by recruiting mitochondria and rough ER-derived vesicles. *L. pneumophila* translocates ~ 300 effectors into the host cell cytoplasm via its type IV secretion system to specifically refine its intracellular niche (Al-Quadan *et al.,* 2012).

Unlike its other intracellular pathogen cousins, the evidence seems to suggest that *L. pneumophila* does not face an amino acid starved milieu. *L. pneumophila* does employ the

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stringent response, which is necessary for survival during infection. In fact, the stringent response in *L. pneumophila* drives the expression of most known virulence factors (Hammer and Swanson, 1999; Molofsky and Swanson, 2004). However, in macrophages, its stringent response machinery does not rely on RelA, suggesting that sensing amino acid starvation is unimportant during infection (Dalebroux *et al.,* 2009; 2010). Instead *L. pneumophila* responds to fatty acid starvation through another stringent response sensor, SpoT, and uses this as a signal to turn on its intracellular pathogenesis programme (Dalebroux *et al.,* 2009). Amino acid catabolism is necessary for growth *in vivo* and some evidence points to serine as a key intracellular carbon source for *L. pneumophila* (Eylert *et al.,* 2010). Finally, a bacterial transporter, PhtA, is used by *L. pneumophila* as a high-affinity threonine transporter and is necessary for growth in cells, showing that at least one amino acid is acquired from the host (Sauer *et al.,* 2005).

The LCV, then, seems to be an amino acid rich environment designed for a hungry bacterium. How then, does *L. pneumophila* manipulate host cells, normally stingy with their amino acids, to serve up amino acids into the LCV? After all, it is likely that amino acid starvation programmes are triggered in host cells infected with *L. pneumophila.* The bacterium has at least two characterized mechanisms of reversing this starvation.

First, *L. pneumophila* upregulates a host neutral amino acid transporter, SLC1A5, during macrophage infection (Fig. 1) Knock-down of the transporter does not affect host cell viability, but limits *Legionella* growth (Wieland *et al.,* 2005). Even when cells were grown in amino acid- rich media, an 80% decrease in SLC1A5 expression resulted in ~ 1000-fold decreased *L. pneumophila* growth (Wieland *et al.,* 2005). The same transporter is specifically upregulated during infection with another intracellular pathogen, *Francisella,* and is also required for bacterial replication in THP-1 cells (Barel *et al.,* 2012). It is unclear whether SLC1A5 exists on the LCV membrane, the plasma membrane, or both, but it is essential in providing amino acids to *L. pneumophila.*

Second, a recent study of host–pathogen interactions at the LCV membrane revealed that *L. pneumophila* utilizes the host proteasome to generate free amino acids for bacterial growth (Price *et al.,* 2011). The authors noted that the LCV is decorated with Lys48-linked polyubiquitinated proteins anchored to the bacterial virulence factor AnkB (Price *et al.,* 2011). These polyubiquitinated proteins recruit the host proteasome, generating small peptides at the LCV membrane. Inhibition of both the proteasome and amino acid-releases peptidases results arrests *L. pneumophila* growth, a phenomenon that is reversed by the addition of excess free amino acids (Price *et al.,* 2011). Using a standard transcriptional reporter of amino acid starvation (green fluorescent protein fused to the *flaA* promoter), the authors found that while wild-type *L. pneumophila* did not experience amino acid starvation during infection – confirming previous stringent response studies – AnkB null mutants did. AnkB growth was not further suppressed by proteasome inhibition, and was rescued by amino acid supplementation. Thus, by recruiting proteins and targeting them for proteasomal degradation, AnkB provides a ready source of amino acids (Price *et al.,* 2011). *L. pneumophila* actively moulds the LCV into a growth-supporting niche, subverting host amino acid starvation machinery and exploiting host AA-acquisition mechanisms (Al-Quadan *et al.,* 2012).

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Concluding remarks

Many of us take a very pathogen-centric view of host defence. However, the vast majority of bacteria encountered by humans are incapable of causing infection. 'Non-specific' defences, such as nutrient deprivation, play a large role in eliminating organisms that are not specifically adapted to a pathogenic niche. Pathogens, therefore, have evolved specific mechanisms to subvert these defences and to take advantage of the nutrient-rich human host. Amino acids, necessary for protein synthesis and, sometimes, as a carbon source, are often depleted in the intracellular environment as a means of starving the pathogen. Pathogen responses range from growth suppression to manipulating host pathways to reverse amino acid starvation.

Understanding the interplay between host and pathogen could prove to be useful in designing new therapies (Escaich, 2008; Rasko and Sperandio, 2010). The three bacteria discussed in this review, *C. trachomatis, L. pneumophila* and *M. tuberculosis* are major human pathogens that, for various reasons, have proven to be difficult to control. All three represent organisms for which new anti-bacterial therapies are needed. By knowing how our immune system tries to kill these bacteria as well as the bacterial evasion strategies, we could target these processes to synergize with the immune system to kill invading pathogens. For example, blocking tryptophan biosynthesis, could increase IFN-γ-mediated killing of *M. tuberculosis*. Amino acid biosynthesis genes could be reasonable drug targets. It is already targeted by many herbicides, and a small molecule used to treat mouse *Pseudomonas aureginosa* infection was shown to have activity against bacterial tryptophan biosynthesis (Epelbaum *et al.,* 1996; Lesic *et al.,* 2007). Furthermore, host tryptophan needs are supplied through the diet, suggesting that, barring off-target effects, small molecules targeting tryptophan synthesis will not be toxic to the host. Alternatively, the susceptibility of *Legionella* to the inhibition of the proteasome suggests that host-directed therapies could alter that balance in virulence (Price *et al.,* 2011; Al-Quadan *et al.,* 2012). Thus, altering both bacterial and host responses critical for amino acid starvation could provide new avenues for the development of therapeutics.

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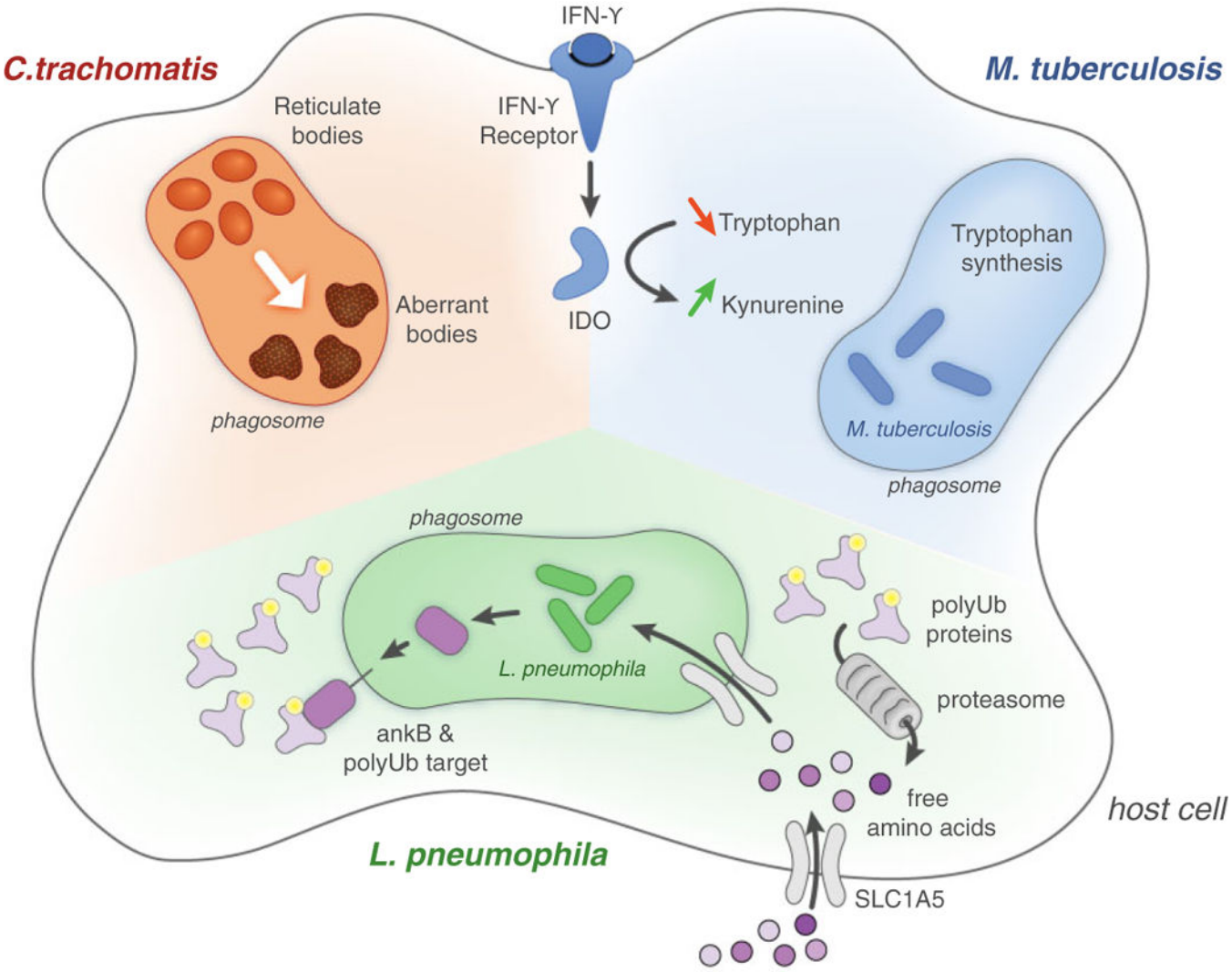

Fig. 1.

Three bacterial strategies for evading amino acid starvation. *Chlamydia trachomatis* responds to amino acid starvation by differentiating to aberrant reticulate bodies, viable but metabolically inactive forms that can differentiate back to active forms when amino acid become available again. While *Mycobacterium tuberculosis* faces amino acid starvation during infection, its constitutive expression of amino acid synthesis makes it generally resistant to host-driven starvation. *Legionella pneumophila* exploits host proteins to override starvation and deliver free amino acids into its intracellular niche. AnkB, a secreted virulence factor, drives the polyubiquitination of LCV membrane proteins. The host proteasome is then recruited to proteolyze ubiquinated targets, producing free amino acids that are transported into the LCV by the upregulated host transporter, SLC1A5.