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Toxoplasma growth *in vitro* is dependent on exogenous tyrosine and is independent of *AAH2* even in tyrosine-limiting conditions

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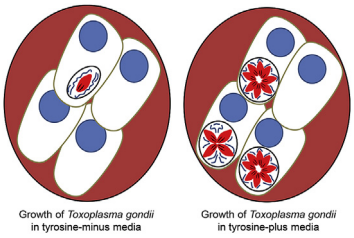
HIGHLIGHTS

- *Toxoplasma* tachyzoites require exogenous tyrosine for growth *in vitro*.
- *Toxoplasma* bradyzoites require exogenous tyrosine for growth but not differentiation *in vitro*.
- AAH2 appears to not be required for tyrosine metabolism in asexual growth of *Toxoplasma gondii in vitro*.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite capable of infecting virtually all nucleated cell types in almost all warm-blooded animals. Interestingly, *Toxoplasma* has a relatively full repertoire of amino acid biosynthetic machinery, perhaps reflecting its broad host range and, consequently, its need to adapt to a wide array of amino acid resources. Although *Toxoplasma* has been shown to be auxotrophic for tryptophan and arginine, it has not previously been determined if *Toxoplasma* is also auxotrophic for tyrosine. *Toxoplasma* tachyzoites and bradyzoites were recently found to express an amino acid hydroxylase (AAH2) that is capable of synthesizing tyrosine and dihydroxyphenylalanine (DOPA) from phenylalanine; however, the role of AAH2 in tachyzoite and bradyzoite infection has not yet been identified. To determine if *Toxoplasma* requires exogenous tyrosine for growth, we performed growth assays on tachyzoites and bradyzoites in nutrient-rich media titrated with varying amounts of tyrosine. We found that *Toxoplasma* tachyzoites form significantly smaller plaques in tyrosine-limiting media in a dose-dependent manner and that this phenotype is not affected by deletion of *TgAAH2*. To determine if bradyzoites require exogenous tyrosine for growth, we induced differentiation from tachyzoites *in vitro* in tyrosine-limiting media and found that replication and vacuole number are all decreased in tyrosine-deficient media. Importantly, culture of confluent human fibroblasts in tyrosine-deficient media does not affect their viability, indicating that, at least *in vitro*, the need for tyrosine is at the level of *Toxoplasma*, not the host cell supporting its growth.

1. Introduction

Toxoplasma gondii is an obligate intracellular Apicomplexan parasite capable of infecting an extraordinarily wide range of cell types in almost all warm-blooded animals. The life cycle of *Toxoplasma* includes a sexual stage, which is only known to occur in the

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feline intestine, and an asexual stage, in which parasites replicate in various intermediate hosts, including humans. Upon infecting an intermediate host, *Toxoplasma* differentiates into tachyzoites that rapidly disseminate throughout host tissues. Although most tachyzoites are cleared by the immune system, some tachyzoites convert to bradyzoites, which form relatively quiescent tissue cysts in the brain and muscle (Dubey, 1998).

Consistent with their parasitic nature, many Apicomplexan parasites have lost some amino acid biosynthetic capabilities; however, the *Toxoplasma* genome encodes a relatively full repertoire of biosynthetic machinery, perhaps reflecting the wide variety of intracellular environments in which it resides (Chaudhary and Roos, 2005; Popp et al., 2015). Computational models of *Toxoplasma* metabolism have successfully predicted many of its metabolic capabilities, such as acetyl-CoA biosynthesis, but have made conflicting predictions on whether tachyzoites are auxotrophic for tyrosine (Chaudhary and Roos, 2005; Song et al., 2013; Tymoshenko et al., 2015). Previous studies have also shown evidence for tachyzoites possessing an active shikimate pathway, which can synthesize tyrosine, tryptophan, and phenylalanine in bacteria; however, *Toxoplasma* does not appear to have orthologs for the enzymes required for *de novo* aromatic amino acid synthesis (Roberts et al., 1998). Moreover, previous studies have shown that depletion of tryptophan in the host cell upon IFN-γ stimulation inhibits growth of *Toxoplasma* tachyzoites, suggesting they are auxotrophic for tryptophan and therefore may not have retained this part of the shikimate pathway (Sibley et al., 1994; Pfefferkorn, 1984).

Recently, two amino acid hydroxylases, *AAH1* and *AAH2*, were found to be expressed at a low level in *Toxoplasma* tachyzoites and upregulated in bradyzoites (Wang et al., 2015). These genes, which have 98% sequence similarity to each other, have homology to both phenylalanine hydroxylase and tyrosine hydroxylase and were shown to be able to convert phenylalanine to tyrosine and tyrosine to dihydroxyphenylalanine (DOPA) (Gaskell et al., 2009). The inclusion of these two genes in computational modeling of *Toxoplasma* metabolism has led to the prediction that tachyzoites are not auxotrophic for tyrosine (Chaudhary and Roos, 2005; Song et al., 2013); however, previous reports have shown that *AAH2* is not required for efficient growth in nutrient-rich media and no role for this gene in the tachyzoite stage or bradyzoite stage has been reported. Attempts to delete *AAH1* using the highly efficient CRISPR-Cas9 have been unsuccessful to date and so its function is unexplored (Wang et al., 2015).

To determine if *Toxoplasma* requires exogenous tyrosine for growth, we performed growth assays on tachyzoites and bradyzoites in nutrient-rich media titrated with varying amounts of tyrosine. Using this approach, we show here that tachyzoites and bradyzoites are indeed auxotrophic for tyrosine but that AAH2 is apparently dispensable for tyrosine metabolism in these two developmental forms.

2. Methods and materials

2.1. Parasite strains, culture, and growth

The *Toxoplasma gondii* Pru*Δku80Δhpt* strain was used for this study. *Toxoplasma* tachyzoites were maintained by serial passage in human foreskin fibroblasts (HFFs) cultured in complete Dulbecco's Modified Eagle Medium (cDMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37 °C in 5% CO_2. Infections included in this study were performed by scraping infected monolayers and lysing the host cells open using a 27 G needle. The released parasites were added to confluent HFFs at the multiplicity of infection (MOI) stated. To measure tachyzoite growth in tyrosine-titrated media, confluent HFFs were washed vigorously three times with phosphate-buffered saline (PBS) and cultured with high-glucose DMEM (Dulbecco and Freeman, 1959) lacking or titrated with varying concentrations of tyrosine and supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The monolayers were immediately infected with tachyzoites in PBS. Plaques were scored by staining cultures with crystal violet after 14 days growth at 37 °C. The number of parasites per vacuole was scored by fixing infected cultures at 36 h post-infection with 4% formaldehyde for 15 min at room temperature (RT). The coverslips were mounted with Vectashield mounting media supplemented with 4,6-diamidino-2-phenylindole (DAPI) stain (Vector Laboratories) and visualized by wide-field fluorescence microscopy. The number of vacuoles and parasites per vacuole were quantified by cytosolic mCherry expression. Unless otherwise noted, only vacuoles with two or more parasites were quantified to be sure extracellular singlet parasites were not incorrectly scored as intracellular.

2.2. Generation of Δaah2 and "wild type" strain

The *TgAAH2* open reading frame (*TgME49_212740*) was deleted from Pru*Δku80Δhpt* and replaced with the hypoxanthine-xanthine-guanine phosphoribosyl transferase (*HXGPRT* or *HPT*) selectable marker by double homologous recombination using the pTKO2-AAH2 vector. To generate the pTKO2-AAH2 vector, ~1.5 Kb and ~1 Kb genomic sequence were amplified by PCR from the 5′- and 3′-regions flanking the *TgAAH2* gene. The 5′-flanking region was amplified using 5′-GCGCGGTACCCACCACACGCAAGGCACTTTC-3′ and 5′-GCGCGAATTCGTGGCCTTATTTGAGCATATTCTG-3′ primer sequences and the resulting fragment was cloned into the KpnI and EcoRI restriction sites of pTKO2 (Caffaro et al., 2013). The 3′-flanking region was amplified using 5′-GCGCAAGCTTCGTTGTGTTCACCGTCGCTAC-3′ and 5′-GCGCGCTAGCGGCGTTTTGACTCTTTTGTATGC-3′ primer sequences and was cloned into HindIII and NheI restriction sites. The pTKO2-AAH2 vector was linearized with NotI, and 30 μg of the linearized plasmid was transfected into Pru*Δku80Δhpt* by electroporation, as previously described. The parasites were allowed to infect HFFs in 24-well plates for 24 h, after which the media was changed to complete DMEM supplemented with 50 μg/ml mycophenolic acid (MPA) and 50 μg/ml xanthine (XAN) for HXGPRT selection. The parasites were passed twice into 24-well plates before being single cloned into 96-well plates by limiting dilution. Deletion of *AAH2* in the *Δaah2* strain was confirmed by PCR using the following primers: 5′-GCAACTTCTCGGGCTCGCGT-3′ and 5′-GATCTTGAGGGAGACAGGAGGCATATGTACAT-3′. The "wild type" control strain was generated by transfecting Pru*Δku80Δhpt* with the pTKO2-AAH2 vector and selecting for stable integration using MPA/XAN, as described above. Stable integration of the vector outside of the *AAH2* locus was confirmed by PCR.

2.3. Viability staining

The viability of human foreskin fibroblasts (HFFs) after continuous culture in tyrosine-free DMEM was determined using the live/dead viability/cytotoxicity kit for mammalian cells (Thermo Fisher). HFFs were cultured in cDMEM on glass coverslips in 24-well plates for a minimum of one week to reach confluency. The cells were then washed vigorously three times with PBS and cultured in DMEM lacking or containing 100 μg/ml tyrosine for 14 days. The cells were then simultaneously stained with green-fluorescent calcein-AM for intracellular esterase activity and red-fluorescent ethidium homodimer-1 for disrupted membrane integrity