Mycobacterium tuberculosis thymidylate synthase gene thyX is essential and potentially bifunctional, while thyA deletion confers resistance to p-aminosalicylic acid

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INTRODUCTION

Thymidylate synthase (TS) enzymes catalyse the biosynthesis of deoxythymidine monophosphate (dTMP or thymidylate), and so are important for DNA replication and repair. Two different types of TS proteins have been described (ThyA and ThyX), which have different enzymic mechanisms and unrelated structures. Mycobacteria are unusual as they encode both thyA and thyX, and the biological significance of this is not yet understood. Mycobacterium tuberculosis ThyX is thought to be essential and a potential drug target. We therefore analysed M. tuberculosis thyA and thyX expression levels, their essentiality and roles in pathogenesis. We show that both thyA and thyX are expressed in vitro, and that this expression significantly increased within murine macrophages. Under all conditions tested, thyA expression exceeded that of thyX. Mutational studies show that M. tuberculosis thyX is essential, confirming that the enzyme is a plausible drug target. The requirement for M. tuberculosis thyX in the presence of thyA implies that the essential function of ThyX is something other than dTMP synthase. We successfully deleted thyA from the M. tuberculosis genome, and this deletion conferred an in vitro growth defect that was not observed in vivo. Presumably ThyX performs TS activity within M. tuberculosis ΔthyA at a sufficient rate in vivo for normal growth, but the rate in vitro is less than optimal. We also demonstrate that thyA deletion confers M. tuberculosis p-aminosalicylic acid resistance, and show by complementation studies that ThyA T202A and V261G appear to be functional and non-functional, respectively.

is converted back to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR; encoded by folA in Escherichia coli and dfrA in Mycobacterium tuberculosis). The more recently discovered ThyX (encoded by thyX), known as the alternative or flavin-dependent TS, also uses mTHF as the methyl donor, yielding THF, but uses reduced flavin adenine dinucleotide (FADH₂) as the reductant (Gattis & Palfey, 2005; Kuhn et al., 2002; Myllykallio et al., 2002). In both cases THF is converted back to mTHF by serine hydroxymethyltransferase.

Most organisms contain either thyA or thyX, and a TS-encoding gene is usually essential (or its deletion creates a thymidine auxotroph if the organism also encodes thymidine kinase). ThyA and ThyX clearly perform the same function, as complementation experiments show that one can functionally replace the other. For example, the thymidine auxotrophy of E. coli lacking thyA can be complemented by thyX from various organisms, including Helicobacter pylori (Myllykallio et al., 2002) and Campylobacter jejuni (Giladi et al., 2002). Also, Halobacterium volcanii thyA can be functionally replaced by Halobacterium salinarum thyX (Giladi et al., 2002). When thyX was deleted from Rhodobacter
capsulatus, both thyA and folA from Rhodobacter sphaeroides were required for full complementation, although thyA alone did partially complement the defect (Leduc et al., 2007). This result is not surprising, as the chemical reaction performed by ThyA requires high DHFR activity (Giladi et al., 2002), and most ThyX-dependent bacteria (including R. capsulatus) do not contain a folA homologue (Leduc et al., 2007; Myllykallio et al., 2002, 2003).

Corynebacteria (including mycobacteria) are unusual as they encode both thyA and thyX (and a folA homologue; Myllykallio et al., 2002), and the biological significance of this is not yet understood. It is known that both thyA and thyX from M. tuberculosis (Rengarajan et al., 2004; Sampathkumar et al., 2005) and Corynebacterium glutamicum (Park et al., 2010) can functionally complement an E. coli thyA deletion strain. ThyA and ThyX from these two species exhibit 72 and 63 % sequence similarity at the amino acid level, respectively (Kan et al., 2010; Park et al., 2010). Both M. tuberculosis ThyA and ThyX proteins have been shown to have TS activity in vitro, with ThyA being substantially more efficient than ThyX (Hunter et al., 2008). However, purified C. glutamicum ThyX does not exhibit any detectable TS activity (Kan et al., 2010). Mutational studies of C. glutamicum show that thyX is not essential for in vitro growth, but is thought to play a role in survival during stationary phase (Park et al., 2010). M. tuberculosis thyA is not thought to be essential, as thyA transposon mutants are viable (Sassetti et al., 2003), and some clinical M. tuberculosis p-aminosalicylic acid (PAS)-resistant strains contain point mutations within thyA (Leung et al., 2010; Mathys et al., 2009; Rengarajan et al., 2004; Zhang et al., 2007). Unlike thyX of C. glutamicum, M. tuberculosis thyX is thought to be essential (Sassetti et al., 2003), and there is pharmacological interest in this enzyme (and ThyX from other pathogenic organisms), as thyX is absent from the human genome (Myllykallio et al., 2002, 2003). Indeed, the structural, functional and kinetic analysis of M. tuberculosis ThyX is already under way so that the rational design of ThyX inhibitors can begin (Hunter et al., 2008; Sampathkumar et al., 2005, 2006; Ulmer et al., 2008). This is of importance, as M. tuberculosis kills approximately two million people annually (WHO, 2009), and no novel antitubercular compound has been introduced since rifampicin in 1963 (Ahmed & Hasnain, 2004). The increasing incidence of infections with multi-drug-resistant (MDR) and extensively drug-resistant (XDR) M. tuberculosis strains, and the associated mortality rates and high cost of second-line antibiotics, heighten the urgent demand for compounds with novel modes of action to treat tuberculosis (Haydel, 2010; Koul et al., 2011; Zignol et al., 2006).

The main aim of this study was to investigate the biological roles of M. tuberculosis thyA and thyX by studying their expression levels, essentiality and roles in pathogenesis, to establish whether either or both of the enzymes that they encode are plausible drug targets.

METHODS

Bacterial strains and culture conditions. E. coli strain DH5α (Invitrogen) was used for all plasmid construction, and strain XL1-Blue (Stratagene) was used for site-directed mutagenesis (SDM). E. coli was grown at 37 °C on Luria–Bertani (LB) agar, or in LB broth with shaking at 250 r.p.m. Where appropriate, E. coli culture media was supplemented with 100 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹, 20 μg gentamicin ml⁻¹ and/or 200 μg X-Gal ml⁻¹.

M. tuberculosis H37Rv was used as wild-type and as the parental strain for deletion strains. M. tuberculosis was usually grown at 37 °C on Middlebrook 7H11 agar (Difco) or in modified Dubos medium (Difco), both supplemented with 4 % Dubos Medium Albumin (Difco) and 0.5 or 0.2 % (w/v) glycerol, respectively. However, 7H11 agar was supplemented with 10 % Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) and 0.5 % (w/v) glycerol when enumerating mycobacterial c.f.u., and the concentration of albumin was increased to 10 % for ΔthyA liquid cultures due to bacterial clumping. M. tuberculosis liquid cultures were grown in a roller incubator at 2 r.p.m. or in an SB3 Tube Rotator (Stewart) at 30 r.p.m. Where appropriate, M. tuberculosis culture medium was supplemented with 25 μg kanamycin ml⁻¹, 15 μg gentamicin ml⁻¹, 50 μg X-Gal ml⁻¹ and/or 20 μg sucrose ml⁻¹.

Stress exposure. M. tuberculosis H37Rv was grown with rolling at 2 r.p.m. to OD₆₀₀ ~0.4, and then 25 ml aliquots were placed into six 50 ml Falcon tubes. For oxidative stress, t-butyl hydroperoxide (TBHP) was added to three cultures to a final concentration of 50 μM, and the other three cultures were untreated controls. For starvation stress, bacteria were harvested by centrifugation, and three cultures were resuspended in 1 x PBS (pH 7.4), while the three control cultures were resuspended in Dubos medium. For nitrosative stress, bacteria were harvested by centrifugation, and three cultures were resuspended in 1 x PBS containing 1 mmol l⁻¹ of sodium nitrite, and the other three cultures were untreated controls. For acid stress, bacteria were harvested by centrifugation, and three cultures were resuspended in acidified Dubos medium (pH 5.4), and the other three cultures were untreated controls. For nitrosative stress, bacteria were harvested by centrifugation, and three cultures were resuspended in acidified Dubos medium (pH 5.4), and sodium nitrite was added to three cultures to a final concentration of 2 mM; the remaining three acidified cultures were controls for nitrosative stress, but also acted as acid-stress cultures compared with the aforementioned control cultures resuspended in non-acidified Dubos medium. Falcon tube cultures were then incubated for 24 h with rotation at 30 r.p.m., and RNA was subsequently prepared.

Murine bone marrow-derived macrophages (BMDM) infection model. Monocytes were isolated from the hind legs of female 6–8 week old BALB/c mice, allowed to differentiate into macrophages for 6 days and flushed from Petri dishes as described elsewhere (Spivey et al., 2011). Macrophages were seeded into either tissue culture treated 75 cm² flasks (for bacterial RNA isolation) or 24-well plates (for bacterial survival assays) at a density of 2.6 x 10⁵ cells per flask or 2 x 10⁵ cells per well. Where appropriate, murine interferon-γ (IFN-γ; Roche) was added at a concentration of 10 ng ml⁻¹. Flasks/plates were incubated at 37 °C with 5 % CO₂ overnight to allow adherence and activation. M. tuberculosis cultures were grown to OD₆₀₀ ~0.5, and washed once with, and then resuspended in, 1 x PBS containing 0.05 % Tween 80 (PBS-T). The cells were centrifuged at 300 r.p.m. to remove any aggregated cells, and an appropriate dilution in PBS-T was prepared. Macrophages were infected at an m.o.i. of either 5:1 in flasks or 0.1:1 in wells for 24 or 4.5 h, respectively. After this time the infected macrophages were washed three times with warm HBSS (Invitrogen), and fresh medium, with or without 10 ng IFN-γ ml⁻¹, was added as appropriate. Flasks/plates were incubated at 37 °C with 5 % CO₂. Mycobacterial RNA was prepared from macrophages in flasks after a further 24 h incubation, i.e. 48 h post-infection (see next section). The survival and multiplication of M. tuberculosis were determined by enumerating c.f.u. within macrophages in wells at various time points.

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RNA purification and quantitative real-time RT-PCR (qRT-PCR) analysis. To isolate mycobacterial RNA from infected BMDM, medium was removed from the flasks and replaced with two volumes (26 ml) of guanidine thiocyanate (GTC) solution (4 M GTC, 25 mM sodium citrate, 0.5 % N-laurylsarcosine sodium salt, 0.5 % Tween 80 and 100 mM β-mercaptoethanol). Cells were scraped from each flask with a cell scraper, the solution was poured into a 50 ml Falcon tube, vortexed vigorously and centrifuged, and RNA was then prepared from the pellet using a FastRNA Pro Blue kit (Qbiogene).

Additional RNA purification, removal of contaminating DNA, conversion to cDNA and qRT-PCR were performed as described previously (Fivian-Hughes & Davis, 2010), except that data were normalized to 16S rRNA.

Plasmid construction. The plasmids used in this study are listed and their construction described in Table 1. Primers are listed in Table 2. SDM was performed using the QuikChange SDM kit (Stratagene). All plasmids were verified by DNA sequencing.

Creating M. tuberculosis deletion strains. The 5’ and 3’ flanking regions of interest were amplified by PCR and were sequentially cloned into the suicide plasmid pBackbone (Gopaul, 2002), using restriction enzymes for which sites were incorporated into the primers. The 6.4 kb sacB/lacZ cassette from pGoal17 (Parish & Stoker, 2000) was cloned into the unique PacI site of the plasmids. Deletion plasmids were electroporated into M. tuberculosis H37Rv, and screening and counter-selection processes were performed as outlined elsewhere (Parish & Stoker, 2000). Primers were designed such that 750 bp of the 792 bp thyA gene was deleted, leaving only the first 32 bp and the last 10 bp, separated by an XbaI site. Likewise, 738 bp of the 753 bp thyX gene was deleted, leaving only the first 4 bp and the last 11 bp, again separated by an XbaI site.

Southern blotting. Putative M. tuberculosis thyA deletion strains were confirmed by Southern blot analysis performed as described previously (Fivian-Hughes & Davis, 2010), except that genomic DNA was digested with PstI, and the probe consisted of a 240 bp PCR product generated from primers thyA probeF and thyA probeR.

Assessing in vitro growth. M. tuberculosis strains were grown with rolling at 2 r.p.m. to OD<sub>600</sub> ~0.5. These cultures were used to inoculate fresh media to OD<sub>600</sub> ~0.02, and the OD<sub>600</sub> was then monitored for 14 days.

Mouse infection studies. M. tuberculosis strains were prepared as for macrophage infections, except that the final resuspension in PBS did not contain Tween 80. Female 6–8 week old BALB/c mice were injected intravenously with approximately 1 × 10<sup>9</sup> bacteria. The survival and multiplication of M. tuberculosis were determined by enumerating c.f.u. within the lungs and spleens of four mice at each time point.

Assessing PAS susceptibility. M. tuberculosis strains were grown with rotation at 30 r.p.m. to OD<sub>600</sub> ~0.4. Cultures were serially diluted in PBS-T and were plated onto agar containing 0, 0.1, 1, 10 or 100 μg PAS ml<sup>−1</sup>. c.f.u. were enumerated and percentage survival relative to untreated samples was calculated.

RESULTS

thyA is expressed at a higher level than thyX

Earlier microarray analysis suggested that M. tuberculosis thyX mRNA was undetectable during both in vitro and in...
**vivo** growth (Talaat et al., 2004). Therefore, we first decided to study *in vitro* expression levels of thyA and thyX using qRT-PCR, which is a more sensitive technique than microarray technology. Both thyA and thyX were detectable by qRT-PCR from *M. tuberculosis* H37Rv grown to exponential phase (Fig. 1a). The expression of both of these genes was significantly reduced upon reaching stationary phase (Fig. 1a), as one might expect when the DNA replication rate is lower, to the point that thyX was undetectable. Interestingly, thyA expression was significantly higher than thyX expression in both growth phases (Fig. 1a; Student’s *t* tests, *P* < 0.05 and *P* < 0.01 for exponential and stationary phase, respectively), yet thyX is the gene predicted to be essential (Sassetti et al., 2003).

It has been postulated that mycobacteria might preferentially use ThyA or ThyX under different growth conditions (Sampathkumar et al., 2005). We therefore assessed thyA and thyX expression after 24 h exposure to various *in vitro* stresses, in search of a condition where thyX expression might be increased. Expression of thyA was significantly decreased by 2.4-fold upon both oxidative and nitrosative stress, and was significantly increased by 2.9-fold upon acid stress, when compared with appropriate controls (Fig. 1b).

Expression of thyX was significantly decreased by 3.2-fold upon starvation, and was significantly increased by 1.6-fold upon acid stress (Fig. 1b). For all of the *in vitro* growth conditions tested, thyA expression remained significantly higher than that of thyX (Fig. 1b; Student’s *t* tests, *P* < 0.01 for all test conditions except TBHP, where *P* < 0.05).

It should be noted that the exponential phase cultures in Fig. 1(a) are essentially the same as the untreated cultures in Fig. 1(b), but thyA expression is significantly lower in Fig. 1(b) (Student’s *t* test, *P* < 0.01). This may be due to the level of culture aeration, as 50 ml cultures were grown in 1 l bottles with rolling at 2 r.p.m. for the experiment shown in Fig. 1(a), while the data shown in Fig. 1(b) were obtained from 25 ml cultures incubated in 50 ml Falcon tubes rotating at 30 r.p.m. (see Methods). It is not known why the level of aeration would have this effect on thyA expression.

**thyA and thyX expression significantly increases during macrophage infection**

To investigate whether thyA or thyX expression is altered *in vivo*, *M. tuberculosis* H37Rv was used to infect murine...
BMDM at an m.o.i. of 5:1. Mycobacterial RNA was isolated 48 h post-infection using a solution containing GTC (see Methods). GTC solution lyses the macrophages and stabilizes the mycobacterial RNA, thus allowing the separation of bacteria from the macrophage lysate without compromising the mycobacterial RNA (Butcher et al., 1998). No thyA or thyX expression was detected during preliminary qRT-PCR experiments using uninfected macrophages (data not shown), indicating either that host RNA was absent or that any contaminating host RNA could not be amplified with the mycobacterial-specific primers. Expression of thyA and thyX was significantly increased within both naive and IFN-γ-activated macrophages, when compared with in vitro exponential expression levels (by 4.0-fold and 4.4-fold for thyA, and 7.9-fold and 8.0-fold for thyX, respectively; Fig. 1c). However, thyA expression remained significantly higher than thyX expression within both naive and activated macrophages (Fig. 1c; Student’s t tests, P<0.05 or P<0.01, respectively).

thyX (Rv2754c) is essential

thyX (Rv2754c) was targeted for in-frame deletion within M. tuberculosis, as RT-PCR indicated that it is co-transcribed with its downstream genes dapA (Rv2753c) and Rv2752c (Fig. 2a; data not shown). After electroporation of pH-thyX into M. tuberculosis H37Rv, potential single cross-over recombinants were analysed by PCR (results not shown), to ensure that integration within the strain taken forward had occurred at the correct locus. Upon selection, no double cross-over strains, i.e. thyX deletion strains, were obtained under normal conditions, or in the presence of 50, 100 or 500 μg thymidine ml⁻¹ (all colonies obtained or 36 colonies were analysed by PCR for each condition; data not shown). This suggests that thyX is essential for the survival of M. tuberculosis, even in the presence of thyA and exogenous thymidine.

To confirm essentiality, an additional copy of thyX expressed from its own promoter (on pASF59) was integrated into the att site of the thyX single cross-over strain, creating a merodiploid strain. Empty vector (pKP203) was also independently integrated into the same single cross-over strain. Upon selection, the wild-type copy of thyX was successfully deleted in the presence of pASF59 (11 out of 36 colonies analysed by PCR), but not in the presence of empty vector (0 out of 36 colonies analysed by PCR), confirming that thyX is essential under the conditions used (Fig. 2b). The addition of 100 μg thymidine ml⁻¹ had no effect on the

![Fig. 1.](image-url) In vitro and in vivo expression levels of thyA and thyX. qRT-PCR was performed on RNA extracts of M. tuberculosis H37Rv (a) grown to exponential and stationary phases (OD₆₀₀ 0.8 and 4.0, respectively), (b) after 24 h exposure to different stress conditions and (c) after 48 h intracellular growth within murine BMDM. The data are means of at least three biological replicates or at least six infections (each assayed in triplicate), normalized to corresponding rrS (16S rRNA) values; error bars, SD. Oxidative stress was modelled using 100 μM TBHP, and untreated cultures acted as controls. Starvation and acid stress were modelled by resuspending cells in PBS or acidified media (pH 5.4), respectively, and cells resuspended in media (Media) acted as control cultures. Nitrosative stress was modelled by resuspending cells in acidified media containing 2 mM sodium nitrite, and cells resuspended in acidified media acted as control cultures. Significant differences in thyA or thyX expression from equivalent exponential phase or appropriate control levels are indicated (* or **; Student’s t test, P<0.05 or P<0.01, respectively).
ability to delete the wild-type copy of thyX from either of these strains (data not shown), supporting the hypothesis that M. tuberculosis cannot utilize exogenous thymidine because its genome does not encode thymidine kinase (Myllykallio et al., 2002).

**thyA (Rv2764c) is not essential**

thyA (Rv2764c) was targeted for in-frame deletion within M. tuberculosis, as RT-PCR indicated that it is co-transcribed with its downstream genes dfrA (Rv2763c) and Rv2762c (Fig. 2a; data not shown). We did not assess whether this transcript extends beyond Rv2762c, so it remains possible that thyA-Rv2757c forms a single operon. Strains containing the thyA deletion were isolated; five of the 28 white, sucrose-resistant, kanamycin-sensitive colonies analysed by PCR gave the products expected for the thyA deletion (results not shown), and three of these were further confirmed by Southern blotting (Fig. 2c, d). M. tuberculosis thyA deletion strain 1 from Fig. 2(d), designated ΔthyA, was used for all further experiments.

**thyA deletion confers an in vitro growth defect**

An immediate growth defect was seen when attempting to grow M. tuberculosis ΔthyA with rolling at 2 r.p.m. in modified Dubos medium [containing 4% albumin and 0.2% (w/v) glycerol]. The strain grew noticeably more slowly than wild-type H37Rv and began to clump at OD600 ~0.5. Upon increasing the albumin concentration within the medium to 10%, ΔthyA no longer clumped and so it was possible to produce growth curves, alongside H37Rv and the complemented strain (ΔthyA containing pASF63). The OD600 of M. tuberculosis ΔthyA differed significantly from that of wild-type H37Rv at four of the seven time points (Fig. 3). M. tuberculosis ΔthyA appears to have a prolonged lag phase compared with wild-type H37Rv, and this growth defect was restored to the wild-type phenotype in the complemented strain (Fig. 3). The addition of thymidine (100 μg ml⁻¹) did not have any effect on the growth of these three strains (results not shown), again supporting the hypothesis that M. tuberculosis cannot utilize exogenous thymidine. From this point onward, all liquid media contained 10% albumin.
**thyA deletion does not affect in vivo growth**

Infection models were utilized to assess the effect of thyA deletion on in vivo growth. *M. tuberculosis* H37Rv and ΔthyA were used to infect murine BMDM (at an m.o.i. of 0.1:1 for 4 h) and immunocompetent BALB/c mice (intravenously). Bacteria within macrophages and within the lungs and spleens of mice were enumerated at various time points post-infection to assess the survival and multiplication of each strain. Although the quantity of *M. tuberculosis* ΔthyA within naïve macrophages differed significantly from equivalent wild-type H37Rv numbers at later time points (Fig. 4a), this was not the case in the activated macrophage and mouse model analysis (Fig. 4a, b). Therefore, we concluded that the deletion of thyA does not affect *M. tuberculosis* in vivo growth.

**thyX expression is not elevated to compensate for the loss of thyA**

As our studies had shown that the expression of thyX was much lower than that of thyA under all conditions tested, yet it was possible to delete thyA we wondered whether the expression of thyX might be elevated in the thyA deletion strain as a means of compensating for its absence. To assess this, the relative expression levels of thyA, dfrA and thyX within *M. tuberculosis* H37Rv, ΔthyA and the complemented strain were quantified by qRT-PCR (Fig. 5). The results confirmed that there was significantly less thyA expression within *M. tuberculosis* ΔthyA when compared with wild-type H37Rv levels, and that thyA expression was restored to wild-type levels within the complemented strain. Furthermore, dfrA expression was unaffected by thyA deletion, so the deletion does not appear to have resulted in downstream polar effects. Importantly, the expression of thyX was also unchanged in the thyA deletion strain. This suggests that although both *M. tuberculosis* ThyA and ThyX proteins are known to have TS activity in vitro (Hunter et al., 2008), thyX expression is not upregulated to compensate for the lack of thyA expression.

**thyA deletion confers PAS resistance**

As some clinical *M. tuberculosis* PAS-resistant strains have mutations within thyA (Leung et al., 2010; Mathys et al., 2009; Rengarajan et al., 2004; Zhang et al., 2007), we investigated whether thyA deletion confers PAS resistance. Briefly, cultures were grown to exponential phase and plated onto agar containing increasing concentrations of PAS (up to 100 μg ml⁻¹). The percentage survival of each strain for each PAS concentration was then calculated compared with that of cells plated without any PAS. The survival of *M. tuberculosis* ΔthyA was significantly greater than that of wild-type H37Rv at all concentrations ≥1 μg PAS ml⁻¹, and this phenotype was restored to wild-type in the complemented strain (Fig. 6). Therefore, thyA deletion confers PAS resistance.

**T202A and V261G mutations, associated with clinical PAS resistance, render ThyA functional and non-functional, respectively**

To further investigate the relationship between ThyA and PAS activity, we analysed the effect of complementing *M. tuberculosis* ΔthyA with thyA containing mutations associated with PAS resistance. The thyA T202V (ACC→GCC) and V261G (GTC→GGC) mutations have been identified within some clinical *M. tuberculosis* PAS-resistant strains, and are thought to affect the structural stability and active catalytic site of the enzyme, respectively (Leung et al., 2010; Mathys et al., 2009; Rengarajan et al., 2004; Zhang et al., 2007). These mutations were independently introduced into the thyA complementation plasmid pASF63 by SDM, yielding pASF70 and pASF71, respectively. *M. tuberculosis* ΔthyA complemented with thyA T202A (pASF70) was able to restore the in vitro growth defect of the deletion strain back to the wild-type phenotype, while the strain complemented with thyA V261G (pASF71) retained the growth defect of the deletion strain (data not shown). In addition, thyA T202A was able to complement the PAS resistance seen for the deletion strain, while thyA V261G could not (Fig. 6). qRT-PCR analysis showed that the introduction of these mutations had no effect on the thyA transcription level from the complementation plasmid (data not shown). Therefore, ThyA T202A appears to be functional and ThyA V261G appears to be non-functional. It seems that functional ThyA is required for *M. tuberculosis* to be sensitive to PAS.
DISCUSSION

To gain insight into the biological roles of *M. tuberculosis* thyA and thyX, we first analysed the in vitro and in vivo expression levels of these two genes by qRT-PCR. Contrary to earlier microarray analysis (Talaat *et al.*, 2004), expression of both thyA and thyX was detected. Under all conditions tested, thyA expression exceeded that of thyX. We were unable to identify a condition where thyX expression alone was increased. However, expression of both genes significantly increased upon in vitro acid exposure (pH 5.4) and when grown within naive and activated murine macrophages, so both ThyA and ThyX may play an important role in vivo. To our knowledge only one other study has shown that thyA is upregulated in vivo (within THP-1 human macrophages; Fontán *et al.*, 2008), while Schnappinger *et al.* (2003) showed that thyA and thyX expression was unchanged in murine macrophages. Again, this illustrates that interesting observations can sometimes be missed when using microarray technology. Microarrays suffer from inherent relatively large variability in expression levels for genes expressed at low levels, meaning that the expression changes of these genes are not shown to be significantly different (Butte, 2002; Chuchana *et al.*, 2007).

In agreement with transposon site hybridization (TraSH) experiments (Sassetti *et al.*, 2003), we showed that *M. tuberculosis* thyX is essential on solid media in vitro. The requirement for thyX in the presence of thyA implies that the essential function of ThyX is something other than dTMP synthase. Earlier studies have indeed questioned whether the true biological substrates of ThyX have been identified, as many of these enzymes (including ThyX from *M. tuberculosis* and *C. glutamicum*) have very low TS catalytic activity (Agrawal *et al.*, 2004; Hunter *et al.*, 2008; Kan *et al.*, 2010). It should be noted that thyX from *C. glutamicum* is only thought to be essential upon reaching stationary phase (Park *et al.*, 2010), and so the biological roles of ThyX appear to differ between these two relatively closely related species. Importantly, we have shown that this enzyme is a plausible tuberculosis drug target, and our collaborators have recently published their findings that compounds specifically designed to inhibit *M. tuberculosis* ThyX are effective, with no activity against ThyA (Kögler *et al.*, 2011). It would be of great interest to identify the

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**Fig. 4.** In vivo growth of *M. tuberculosis* ΔthyA. Growth of *M. tuberculosis* H37Rv and ΔthyA was assessed within (a) murine BMDM (alongside the complemented strain; ΔthyA containing pASF63) and (b) BALB/c mice. The data are means of three macrophage infections or the lungs and spleens of four mice; error bars, sd. Times when the quantity of *M. tuberculosis* ΔthyA was significantly different from that of wild-type H37Rv are indicated (* or **; Student’s *t* test, *P* < 0.05 or *P* < 0.01, respectively).
primary essential function of ThyX in *M. tuberculosis*, and this could possibly be achieved using a metabolomics approach (de Carvalho et al., 2010).

We successfully deleted *thyA* from the *M. tuberculosis* genome, and this deletion was shown to confer an *in vitro* growth defect that was not observed *in vivo*. *M. tuberculosis* has evolved to grow within its human host, and culture medium is a very unnatural environment for the bacterium. The murine macrophage and animal models used within this study are much more representative of the natural *M. tuberculosis* environment. The *in vitro* experiments do support the hypothesis that *M. tuberculosis* cannot utilize exogenous thymidine because its genome does not encode thymidine kinase (Myllykallio et al., 2002). It therefore appears as though ThyX performs TS activity within *M. tuberculosis ΔthyA* at a rate sufficient for normal growth *in vivo*, but that the rate *in vitro* is less than optimal. This could be due to a change in the *thyX* transcription rate and/or ThyX TS activity. We have already shown that *M. tuberculosis thyX* mRNA levels are higher *in vivo* than *in vitro*. Also, *Campylobacter jejuni* ThyX is known to be inhibited by oxygen (Giladi et al., 2002), so *M. tuberculosis* ThyX may be more active *in vivo* under conditions of limited oxygen availability than *in vitro*. The *in vitro* expression level of *thyX* is unchanged within *M. tuberculosis ΔthyA*, indicating that the transcriptional regulation of the two TS genes is probably not linked. It would be interesting to know whether *thyX* is actually essential for *in vivo* growth. This could be investigated by switching (Pashley & Parish, 2003) the existing complementation plasmid within a strain where wild-type *thyX* was successfully deleted, with a plasmid expressing *thyX* from a regulated promoter. The strain could then be grown *in vitro* with *thyX* being expressed and subsequently used to infect macrophages or mice without *thyX* expression (Boldrin et al., 2010; Carroll et al., 2005; Guo et al., 2007). Unfortunately, these experiments are beyond the scope of this study.

Some clinical *M. tuberculosis* PAS-resistant strains contain point mutations within *thyA* (Leung et al., 2010; Mathys et al., 2009; Rengarajan et al., 2004; Zhang et al., 2007). We have conclusively demonstrated that *thyA* deletion confers *M. tuberculosis* PAS resistance. We also showed that ThyA T202A appears to be functional and ThyA V261G appears to be non-functional. This finding is supported by a recent study which demonstrated that the *thyA* T202A mutation is a marker for the Latin American Mediterranean lineage of *M. tuberculosis* and is not associated with PAS resistance (Feuerriegel et al., 2010). The mode of action of PAS is currently unknown. PAS has structural similarities to sulphonamides, which act as competitive inhibitors of dihydropteroate synthase (FolP1 and FolP2), and dihydropteroate is a precursor of DHF. However, PAS appears to be a poor inhibitor of *M. tuberculosis* FolP1 *in vitro* (Nopponputh et al., 1999), and mutations within *M. tuberculosis* folP1 and folP2 have not been found to be associated with PAS resistance (Mathys et al., 2009). It has been suggested that PAS may be a pro-drug whose activation requires viable ThyA (Mathys et al., 2009;
Rengarajan et al., 2004), but only approximately 36% of clinical and spontaneous M. tuberculosis PAS-resistant strains have mutations in thyA (Mathys et al., 2009; Zhang et al., 2007). Also, Mycobacterium bovis BCG thyA mutations confer resistance to 8710, a trimethoprim analogue that antagonizes mycobacterial DHFR, in addition to PAS (Rengarajan et al., 2004). Therefore, PAS appears to target an unknown component of folate biosynthesis, and as ThyA is a major consumer of THF, strains mutated in thyA are able to withstand the pressure on the folate cycle (Leduc et al., 2007; Rengarajan et al., 2004). Nevertheless, thyA sequencing has proved effective at predicting PAS treatment outcome (Leung et al., 2010).

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