Analysis of the contribution of MTP and the predicted Flp pilus genes to *Mycobacterium tuberculosis* pathogenesis

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*Mycobacterium tuberculosis* (Mtb) is one of the world’s most successful pathogens. Millions of new cases of tuberculosis occur each year, emphasizing the need for better methods of treatment. The design of novel therapeutics is dependent on our understanding of factors that are essential for pathogenesis. Many bacterial pathogens use pili and other adhesins to mediate pathogenesis. The recently identified *Mycobacterium tuberculosis* pilus (MTP) and the hypothetical, widely conserved Flp pilus have been speculated to be important for Mtb virulence based on *in vitro* studies and homology to other pili, respectively. However, the roles for these pili during infection have yet to be tested. We addressed this gap in knowledge and found that neither MTP nor the hypothetical Flp pilus is required for Mtb survival in mouse models of infection, although MTP can contribute to biofilm formation and subsequent isoniazid tolerance. However, differences in *mtp* expression did affect lesion architecture in infected lungs. Deletion of *mtp* did not correlate with loss of cell-associated extracellular structures as visualized by transmission electron microscopy in Mtb Erdman and HN878 strains, suggesting that the phenotypes of the *mtp* mutants were not due to defects in production of extracellular structures. These findings highlight the importance of testing the virulence of adhesion mutants in animal models to assess the contribution of the adhesin to infection. This study also underscores the need for further investigation into additional strategies that Mtb may use to adhere to its host so that we may understand how this pathogen invades, colonizes and disseminates.

**INTRODUCTION**

*Mycobacterium tuberculosis* (Mtb) is one of the leading infectious causes of death worldwide, resulting in 9.6 million new tuberculosis (TB) cases and 1.5 million deaths in 2014 (WHO, 2015). High rates of TB disease and multi-drug resistance emphasize the need for better therapeutics to combat this deadly pathogen. The design of new drugs will be informed by our understanding of the factors required for Mtb pathogenesis.

Adhesins, which are cell surface molecules used for adherence, are essential for the virulence of many bacterial pathogens (Barocchi et al., 2006; Connell et al., 1996; Khelef et al., 1994; Mulvey et al., 2001; Nielsen et al., 2012; Schreiner et al., 2003; Taylor et al., 1987; Terao et al., 2012). Bacterial adhesins play roles in attachment, entry, invasion (Mandlik et al., 2008; Pizarro-Cerdà et al., 2006), tropism (Wright & Hultgren, 2006) and colonization of host cells (Mandlik et al., 2008). In addition, adhesins are often important for biofilm formation (Flores-Mireles et al., 2015; Foster et al., 2014; Mandlik et al., 2008; Telford et al., 2006), signal transduction (Moorthy et al., 2016), immune activation (Blanco et al., 2012; Lee et al., 2005; Mandlik et al., 2008) and immune evasion (Flores-Mireles et al., 2015; Foster et al., 2014; Nobbs et al., 2009).

While several Mtb proteins have been classified as adhesins [recently reviewed in Govender et al. (2014)], their functional roles are often largely unclear. In particular, two types of pili have been recently reported in Mtb. Pili were initially investigated in Gram-negative bacteria, and it was long
thought that Mtb did not express a pilus. Several years ago, it was reported that the Mycobacterium tuberculosis pilus (MTP) (encoded by Rv3312a) is present in multiple strains of Mtb (Alteri et al., 2007). MTP binds laminin, can be recognized by immune sera from active TB patients (Alteri et al., 2007) and resembles curli, which are extracellular proteinaceous fibres produced by many Enterobacteriaceae that share biochemical properties with amyloid (Barnhart & Chapman, 2006; Blanco et al., 2012). The potential importance of MTP as a Mtb virulence factor and biomarker (Naidoo et al., 2014) has spurred research from multiple groups and has led to a number of reviews on mycobacterial adhesins (Govender et al., 2014; Hosseini et al., 2014; Ramsugit & Pillay, 2015). Recent studies using a clinical isolate of Mtb, V9124, have shown that an mtp deletion mutant is defective in in vitro pellicle biofilm formation (Ramsugit et al., 2013) and in adherence to and invasion of A549 epithelial cells (Ramsugit et al., 2016), but it has no defect in adhering to and invading THP-1 macrophages (Ramsugit & Pillay, 2014). These same studies also show that a V9124 mtp complemented overexpression strain forms normal biofilms (Ramsugit et al., 2013), adheres to and invades A549 epithelial cells similarly to WT bacteria (Ramsugit et al., 2016) and has enhanced invasion and adherence to THP-1 macrophages (Ramsugit & Pillay, 2014). However, the biological relevance of MTP during Mtb pathogenesis has not been directly tested, and it should be noted that the characterization of MTP as an extracellular structure is limited to two publications (Alteri et al., 2007; Ramsugit et al., 2013).

In addition to the mtp gene, the genome of Mtb also contains the tad (tight adherence) operon, which encodes the components of a type IV pilin assembly system known as the Flp pilus, named for its major structural pilin protein Flp (Kachlany et al., 2000). The tad locus is widely distributed in many bacterial and all archaeal genomes and was originally identified in the Gram-negative oral pathogen Aggregatibacter actinomycetemcomitans, where it is essential for tight adherence, autoaggregation, rough colony morphology and virulence (Kachlany et al., 2001; Planet et al., 2003; Schreiner et al., 2003). Although the tad operon consists of 14 genes in A. actinomycetemcomitans, Mtb has only maintained five genes of the tad operon: tadZ, encoding an inner membrane-associated cytoplasmic ATPase-like protein potentially involved in pilin localization; tadA, encoding an inner membrane ATPase that drives pilin assembly; tadB and tadC, encoding proteins that may serve as a secretion apparatus and the major flp pilin gene (Tomich et al., 2007). Additionally, two predicted pseudopilin genes adjacent to the tadZABC-flp locus in Mtb encode proteins with partial homology to TadE and TadF in A. actinomycetemcomitans. However, the proteins encoded by these genes are missing the conserved N-terminal G/XXXXEF motif found in other pseudopilins (Kachlany et al., 2000) and, therefore, have been disregarded as true members of the tad system in Mtb. It has been speculated that this predicted Flp pilus could contribute to Mtb pathogenesis (Alteri, 2005; Ramsugit & Pillay, 2015), but this has not been investigated.

Despite the recent attention that MTP and the predicted Flp pilus have gained, roles for Mtb pili in pathogenesis thus far have only been speculated. In this study, we address this gap in knowledge by testing the virulence of Mtb Erdman strain mtp and flp mutants in mouse models of infection. Importantly, we also examine whether phenotypes of these mutants correlate with loss of cell-associated extracellular structures resembling pili.

### METHODS

#### Bacterial strains and growth conditions

Mtb strains Erdman and HN878 and their derivatives were cultured at 37 °C in Middlebrook 7H9 (broth) or Middlebrook 7H10 (agar) supplemented with 10% oleic acid/albumin/dextrose/catalase, 0.5% glycerol and 0.05% Tween 80 (7H10 only) or in Sauton’s liquid medium. When needed, Congo red (CR; Sigma) was added to 7H10 plates at a concentration of 100 µg ml⁻¹ (Parrish et al., 2004). Bacterial biofilms were inoculated with stationary phase planktonic cultures into Sauton’s medium at a 1:100 dilution. Culture vessels were closed tightly to restrict oxygen for 3 weeks and then vented as previously described (Ojha et al., 2008). Rugose colony biofilms were formed by pipetting 5 µl of stationary phase planktonic Mtb on agar plates. In mycobacterial cultures, 20 µg ml⁻¹ kanamycin, 50 µg ml⁻¹ hygromycin and isoniazid (INH) at indicated concentrations were supplemented as needed.

#### Construction of mutant strains

For the creation of the Mtb Δmtp strains in the Erdman and HN878 background, a specialized transducing phage containing homology to the Mtb H37Rv reference genome nucleotides 3701044 to 3701747 and 3700108 to 3700707 was used to replace the endogenous mtp gene with a hygromycin resistance cassette. Mutants were confirmed by Southern blotting. The Δmtp::mtp complemented strain that constitutively expresses mtp was created by integrating pMSG430 mtp into the attB site of the Δmtp strain. The Δmtp::attP/mtp vector control strain was created by integrating pMSG430 into the attB site of the Δmtp strain (see Fig. S1, available in the online Supplementary Material).

Creation of strains deficient in various components of the tad locus/Flp pilus was carried out in the same way. Specialized transducing phage containing homology to Mtb nucleotides 4097979 to 4098656 and 4095482 to 4095482 was created for the deletion of tadA and tadB, while phage containing homology to Mtb nucleotides 4096132 to 4096762 and 4094636 to 4095323 was used for the deletion of tadC and flp.

#### Crystal violet staining

Mtb biofilm biomass was quantified by adapting previously published protocols (O’Toole & Kolter, 1998). Cultures were grown under pellicle biofilm-forming conditions in 96-well plates, media were aspirated and plates were gently washed with water three times. Plates were stained with 0.5% crystal violet for 15 min, washed three times in water and air dried. To quantify staining, we used 45% acetic acid to de-stain each well, diluted at 1:10 in formalin and read at OD570.

#### Stress and tolerance assays

Mtb was grown under biofilm-forming conditions in 24-well plates in Sauton’s medium. After 3 weeks, samples on the vessels were opened and concentrated solutions of INH or water control were pipetted underneath the surface of the culture. After 2 weeks of exposure to the indicated stress, bacteria were harvested from each well, centrifuged to pellet and resuspended in 1% Tween 80 in PBS. Glass beads were added to each tube and tubes were shaken overnight at room temperature to disassociate bacteria. Serial dilutions were...
**Fig. 1.** *mtp* transcript levels. *mtp* transcript levels in *Mtb* HN878 WT and Erdman WT logarithmic (log) and stationary planktonic cultures, as well as 2- and 3-week (wk) Erdman biofilm (BF) cultures were measured by qRT-PCR, normalized to *sigA* transcript levels and expressed as a fold change from Erdman log phase cultures. Each bar represents triplicate biological replicates except 3-week BF, which represents duplicate biological replicates. Graphical data in this and subsequent figures are represented as mean±SEM. Statistical differences were determined by one-way ANOVA and Tukey’s multiple comparison test. *P*<0.05, **P**<0.01, ***P***<0.001 and ****P***<0.0001; NS, not significant.

Plated to enumerate c.f.u. For planktonic stress tolerance assays, bacteria were cultured into 7H9 medium containing 0.05% Tween 80 and grown for 14 days in 96-well plates at 37°C. After 14 days, bacteria were harvested and serial dilutions were plated to enumerate c.f.u. and determine survival.

**Quantitative real-time PCR.** RNA was isolated from mycobacteria using Trizol (Invitrogen) and chloroform followed, by either isopropanol and high salt precipitation or extraction with the Direct-Zol RNA Miniprep (Zymo Research). DNA was removed using the TURBO DNA-free kit (ThermoFisher Scientific), cDNA was prepared using Superscript III (Invitrogen) and quantitative real-time PCR (qRT-PCR) was performed using iTag Universal SYBR Green Supermix (Bio-Rad). Primers used to amplify *mtp* were 5'-TGCAGACTATCCTGTTGCC-3' and 5'-CAGGGACTTTCGAGATGG-3'. Levels of *mtp* transcript were normalized to *sigA* transcript levels as previously described (Stallings et al., 2009).

**Negative staining and analysis by electron microscopy.** Biomass from *Mtb* rugose colony biofilms or pellicle biofilms was collected into 4% paraformaldehyde (Electron Microscopy Sciences) and vortexed. Samples were allowed to absorb onto glow-discharged formvar/carbon-coated copper grids. Grids were washed in distilled water and stained with 1% aqueous uranyl acetate (Ted Pella) for 1 min. Excess liquid was gently wicked off and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (TEM; JEOL) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques).

**Mouse infections.** Before infection, exponentially replicating *Mtb* strains were washed in PBS+0.05% Tween 80 and were sonicated to disperse clumps. Female C3HeB/FeJ or C57Bl/6 mice 8–9 weeks old (Jackson Laboratory) were exposed to 8x10⁶ c.f.u. of the appropriate strain in a Middlebrook Inhalation Exposure System (Glas-Col), which delivers ~100 bacteria per animal. Bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto 7H10 agar plates. Plates were incubated at 37°C in 5% CO₂ for 3 weeks prior to counting colonies. For histology, mouse lungs were fixed and stored in 10% buffered formalin until processing. Lungs were then dehydrated using ethanol and processed by the Department of Internal Medicine-Pulmonary Disease Pulmonary Morphology Core, where they were paraffin embedded and sectioned, and consecutive sections were stained with either haematoxylin and eosin (H&E) stain or acid-fast stain. Slides were visualized using an Olympus BX51 light microscope (Olympus) equipped with a MicroPublisher 5.0 digital camera (Q Imaging).

All mice in this manuscript survived unless humanely sacrificed to measure bacterial burden and histological signs of disease. All procedures involving animals were conducted following the National Institutes of Health guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of Washington University in St. Louis School of Medicine (protocol no. 20130156, Analysis of Mycobacterial Pathogenesis). Washington University is registered as a research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care. The Animal Welfare Assurance is on file with the National Institutes of Health’s (NIH) Office for Protection from Research Risks. All animals used in these experiments were subjected to no or minimal discomfort. All mice were euthanized by CO₂ asphyxiation, which is approved by the American Veterinary Association Panel on Euthanasia.

**RESULTS**

*mtp* expression changes based on growth phase

To investigate when MTP may be functioning in *Mtb*, we first monitored *mtp* expression at different phases of bacterial growth in two different strains of *Mtb*, the laboratory strain Erdman of the Euro-American lineage and the hypervirulent HN878 strain of the W-Beijing lineage. We found that levels of *mtp* transcript were upregulated 14-fold in stationary phase Erdman cultures and 22-fold in stationary HN878 cultures relative to logarithmic phase Erdman cultures (Fig. 1). This growth phase-dependent expression of *mtp* in both strains suggests that the function of MTP is more important for *Mtb* in stationary phase compared to logarithmic phase. The levels of *mtp* in logarithmic phase were similarly low in Erdman and HN878 (with average Ct values of 25.3 and 26.4 in qRT-PCR analyses, respectively), but the induction of *mtp* in response to stationary phase was significantly more robust in HN878, suggesting that MTP may play a greater role in stationary phase in the HN878 strain (Fig. 1). In other bacterial pathogens, pili and curli are known to contribute to biofilm formation (DePas...
mtp expression has strain-specific effects on biofilm formation

mtp expression in biofilms and the established contribution of pili and curli to biofilm formation in other bacteria led us to explore whether MTP plays a role in biofilm formation in Mtb Erdman and HN878 strains. To investigate a potential role for MTP in Mtb biofilm formation, we first engineered the genetic mutants Δmtp, Δmtp+mtp complemented strain and Δmtp-empty vector control strain in the Mtb Erdman background (Fig. S1). The Δmtp mutant was confirmed by Southern blot analysis (Fig. 2a). WT or mtp mutant strains of Mtb were inoculated into Sauton’s medium and culture vessels were closed tightly to restrict oxygen for 3 weeks prior to venting, after which the pellicle biofilm robustly developed at the air–liquid interface (performed similarly as in Ojha et al., 2008). We found that mtp transcripts were upregulated in 2-week but not in 3-week biofilm cultures relative to logarithmic phase planktonic cultures (Fig. 1).

mtp expression does not correlate with the abundance of cell-associated extracellular structures visualized by TEM

The absence of effects on CR binding raised the question of the presence and the nature of MTP curli-like pili, which have not been confirmed across the field of Mtb research and have only been studied microscopically in two publications (Alteri et al., 2007; Ramsugit et al., 2013). The structures referred to as the curli-like MTP in each of these publications appear morphologically diverse within a single paper and across the two publications. Alteri et al. (2007) reported that 10% of mycobacterial cells (from H37Rv, H37Ra and CDC1551 strains) produced MTP structures, while Ramsugit et al. (2013) reported that 80% of WT V9124 were ‘piliated’. Therefore, in order to determine

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**Fig. 2.** Biofilm formation of mtp deletion strains. (a) Southern blot analysis of WT and mtp deletion mutants in Erdman and HN878 strains. Genomic DNA was digested with EcoRV, which yields a 6.3 kb WT band or a 2.3 kb Δmtp band. (b) Pellicle
biofilm formation of Erdman WT, Δmtp, Δmtp+mtp, Δmtp+empty, HN878 WT and HNΔmtp strains in 24-well plates. (c) Crystal violet quantification of pellicle biofilm formation from 96-well plates. Each bar represents triplicate data across three independent experiments. (d) Rugose colony biofilm morphology of Erdman WT, Δmtp, Δmtp+mtp,Δmtp+empty, HN878 WT and HNΔmtp on CR 7H10 plates. (e) Representative negative staining TEM images from each strain to illustrate the cell-associated structures observed. Scale bars represent 500 nm. (f) Percentage of cells scored as ‘MTP-positive’ across the six bacterial strains by nine blinded reviewers. Each coloured symbol represents a reviewer. The n numbers displayed above each sample dataset correspond to the total number of individual cells for each strain that were scored. Statistical significance was analysed by ANOVA and Tukey’s multiple comparison test. *P<0.05, **P<0.01 and ***P<0.001; NS, not significant.

whether our panel of mutants differed in the presence of curli-like, cell-associated extracellular structures, we performed uranyl acetate negative staining followed by TEM of samples from pellicles and rugose colony biofilms (Fig. 2e, f). Due to controversy in the field over the existence of curli-like MTP and the scarce characterization of MTP itself, we employed nine individual blinded reviewers to score 130 electron microscopic images across the six WT and mutant strains for what we termed ‘MTP-positive’ cells. Reviewers were trained to look for ‘MTP-positive’ cells based on the aggregative, string-like, cell-associated extracellular structures found in figures in the two previous publications (Alteri et al., 2007; Ramsugit et al., 2013). Examples of what would have scored as ‘MTP-positive’ cells from each strain are presented in Fig. 2e. Fig. 2f displays the percentage of cells scored as ‘MTP-positive’ in each strain by each of the nine reviewers, with each reviewer represented by a certain colour symbol. Fig. 2f demonstrates that even with variability in what is considered ‘MTP-positive’ across reviewers, the three strains that are genetically deficient for mtp (Δmtp, Δmtp-empty, HNΔmtp) are still viewed as having ‘MTP-positive’ cells based on the presence of cell-associated extracellular structures similar to those reported in previously published work. The only statistically significant comparisons are with Δmtp-empty, which on average has relatively lower reports of ‘MTP-positive’ cells but still had examples of cell-associated extracellular structures. These data show that phenotypes associated with these strains correlate with the presence, absence or constitutive expression of mtp but do not correlate with differences in the abundance of cell-associated extracellular structures.

Constitutive expression of mtp enhances tolerance to INH

Biofilm cultures of Mtb harbour increased numbers of antibiotic-tolerant bacteria relative to planktonically grown Mtb (Ojha et al., 2008). To investigate whether the different pellicle biofilm phenotypes in the Erdman WT, Δmtp, Δmtp+mtp and Δmtp-empty strains (Fig. 2) would affect antibiotic tolerance, we performed a biofilm stress assay with INH. Biofilm cultures were started and carried out as usual for 3 weeks. Upon aeration at the 3-week time point, 50 µg ml⁻¹ INH, 100 µg ml⁻¹ INH or water control was added to the culture by pipetting underneath the premature pellicle (Fig. 3a). Bacteria were harvested from each well after 2 weeks of INH treatment (after a total of 5 weeks under biofilm culturing conditions) and plated for c.f.u. Relative to the untreated control for each strain, significantly more bacteria survived 50 µg ml⁻¹ and 100 µg ml⁻¹ INH treatment in the Δmtp+mtp strain than any of the other strains (Fig. 3b). This difference in survival between strains after INH treatment was not observed in planktonic stress assays (Fig. S2e), indicating that the increased tolerance to INH in the Δmtp+mtp strain is specific to Mtb grown under the biofilm condition. This could be due to either the enhanced biomass of the Δmtp+mtp pellicle itself or MTP providing some other benefit under the nutrient-poor, slow-growing condition of the biofilm. These data together demonstrate that while loss of MTP does not sensitize Mtb to INH, the constitutive expression of MTP can enhance drug tolerance of Mtb grown under biofilm conditions.

MTP is not required for Mtb survival but can impact histopathology in a mouse model of infection

Mtb growing in biofilms share many characteristics with Mtb growing in the host during chronic infection, including decreased replication rates, decreased nutrient availability and increased stress tolerance as seen by an increase in persister cell formation relative to planktonic culture (Ojha et al., 2008; Richards & Ojha, 2014). Multiple groups have also reported the observation of extracellular communities of Mtb that resemble biofilms during infection (Lenaerts et al., 2007; Orme, 2014; Wong & Jacobs, 2016), although this is highly debated. These necrosis-associated extracellular clusters (NECs) of Mtb were initially observed in guinea pigs in an extracellular microenvironment that is present at the acellular rim of residual primary lesion necrosis (Lenaerts et al., 2007). Lung lesions in the guinea pig model display necrosis, mineralization and hypoxia, and thus are more similar to human lesions than those found in the C57Bl/6 mouse model (Lenaerts et al., 2007). As an alternative to C57Bl/6 mice, populations of necrosis-associated extracellular bacteria in micro-environments similar to the guinea pig model have been found in C3HeB/FeJ mice, which form lesions that are both hypoxic and necrotic (Driver et al., 2012; Harper et al., 2012). In humans, necrosis leads to pulmonary cavitation, which is a hallmark of the most common form of TB (Wong & Jacobs, 2016).

The growing interest in understanding the contribution of biofilm-like NECs to Mtb virulence in vivo, our findings that MTP contributes to biofilm formation and drug...
tolerance (Figs. 2 and 3) and the multiple studies demonstrating attachment defects of *mtp* mutants in *vitro* (Alteri et al., 2007; Ramesugit & Pillay, 2014; Ramesugit et al., 2016) together begged the question of whether MTP contributes to Mtb virulence in animal models. To investigate the role of MTP in Mtb pathogenesis, we infected C3HeB/FeJ mice, (*mtp*+*mtp* strain was not attenuated at any time point in the lung or spleen. In fact, at 56 days post-infection (d.p.i.), the strain had significantly higher c.f.u. in both organs compared to the other two strains. These data indicate that losing *mtp* expression does not hinder bacterial survival in the C3HeB/FeJ mouse model of infection. Furthermore, the *Δmtp* strain showed no statistical difference in bacterial burden relative to WT Mtb. Therefore, despite the increased pellicle formation and subsequent INH tolerance of the *Δmtp* strain, this does not confer a fitness advantage in the mouse. We also found similar c.f.u. trends when we infected C57Bl/6 mice (Fig. S3), supporting that MTP is not required for Mtb colonization, spread or survival in mice.

In addition to monitoring bacterial burden, lungs were also collected for histological analysis. Lungs from C3HeB/FeJ mice at 56 d.p.i. with Erdman WT, *Δmtp* or *Δmtp+mtp* were processed and two consecutive sections were stained for H&E or acid-fast bacilli (Fig. 4c–q). We found extracellular acid-fast -positive bacteria present in lungs of all the mice examined. Two types of lesion were found in the lungs of C3HeB/FeJ mice. The first and more common type of lesion is very inflamed but is less structured, and it contains both intracellular and extracellular bacteria (Fig. 4e, h and k). This type of unencapsulated lesion was present in every lung section from each of the different Mtb strain infections (Fig. 4c–k). The second type of lesion we observed contained distinct margins and has previously been referred to as an encapsulated lesion (Driver et al., 2012) (Fig. 4l–q). The encapsulated lesions were only present in the lungs infected with Erdman WT or *Δmtp* strains. In addition to intracellular bacteria, encapsulated lesions were full of extracellular bacteria, which were often present in clusters (Fig. 4n, q). The prevalence of encapsulated lesions corresponded to the level of bacterial burden at that time point. The strain with the fewest c.f.u. at this 56 d.p.i. time point, *Δmtp+mtp*, had no lungs that contained encapsulated lesions (n=12, 0 %). The Erdman WT strain, which had intermediate c.f.u. levels at the 56 d.p.i. time point, had one lung section that contained one encapsulated lesion (n=12, 8.3 %). *Δmtp* infection led to the highest bacterial burden at 56 d.p.i. and resulted in sections from two separate lungs that each contained two encapsulated lesions (n=6, 33 %). These studies confirm the presence of clustered extracellular bacteria in the C3HeB/FeJ mouse model of TB for all Erdman strains tested, and suggest that the expression levels of *mtp* may influence histopathological features. However,
since the presence of encapsulated lesions correlated not only with mtp expression but also with bacterial burden, the source of the phenotype is uncertain. Therefore, it is possible that mtp expression levels impact immune responses to the infection; however, the finding that MTP is not required for Mtb Erdman infection, spread and survival

**Fig. 4.** Pathogenesis of mtp mutants in C3HeB/FeJ mice. Bacterial titres in the (a) lungs and (b) spleens of C3HeB/FeJ mice infected with Erdman WT (black filled circles), Δmtp (grey filled triangles) or Δmtp+mtp (black filled squares connected by a broken line) strains by the aerosol route. The Erdman WT and Δmtp+mtp infections were performed twice with two or three mice per time point per infection. The data represent the average of two experiments. The Δmtp infection was performed once with three mice per time point. Statistical significance across groups determined by ANOVA, *P < 0.05 and ***P < 0.001. (c–q) Histology of lungs from 56 d.p.i. with Erdman WT (c–e and l–n), Δmtp (f–h and o–q) and Δmtp+mtp (i–k). Consecutive sections were stained with either H&E (c, f, i, l and o) or a stain for acid-fast bacilli (AFB) (d–e, g–h, j–k, m–n and p–q) and visualized at either the ×10 objective (c, d, f, g, i, j, l, m, o and p) or the ×100 objective (e, h, k, n and q) on a light microscope. The top three rows represent the more prevalent, less-structured, unencapsulated lesions found during all infections. The fourth row depicts an encapsulated lesion from Erdman WT infection, while the last row depicts an encapsulated lesion from Δmtp infection.
is contrary to what was predicted from in vitro adherence studies (Ramsugit et al., 2016).

The predicted Flp pilus is not required for biofilm formation or Mtb survival in C57Bl/6 mice

With an interest in characterizing the role of potential pili in Mtb, we generated two Mtb Erdman genetic deletions targeting components of the tad operon, which encodes the predicted type IV Flp pilus (ΔtadAB and ΔtadC-flp; Fig. 5a). Mutant strains were confirmed via Southern blot analysis (Fig. 5b). We used these mutants to interrogate the contribution of the tad operon to Mtb physiology and virulence. Since tad-encoded Flp pilus in other bacteria are required for adherence and autoaggregation (Planet et al., 2003), we sought to assess the ability of Mtb tad mutants to form biofilms. Cultures of WT or tad mutant strains of Mtb were used to inoculate biofilm cultures as described before. No differences were observed in pellicle biofilm development of the tad mutants compared to the WT control (Fig. 5c), demonstrating that the proteins encoded by the tad operon do not play a role in biofilm formation under these conditions.

Given that type IV pili in other bacteria have been associated with host cell adhesion and virulence (Craig et al., 2004; Schreiner et al., 2003), we wanted to investigate the potential contribution of the tad locus to Mtb virulence. To do this, we infected C57Bl/6 mice with Erdman WT, ΔtadAB or ΔtadC-flp strains of Mtb and measured pulmonary and splenic Mtb burden at various time points post-infection. Neither of the Mtb tad mutant strains was attenuated in this model (Fig. 5d, e). The number of Mtb c.f.u. in the lungs and spleens of infected mice showed no statistically significant differences between the strains tested across all time points, except at 35 d.p.i. when ΔtadC-flp had a small but significant increase in bacterial burden in the lung (Fig. 5d). The unattenuated colonization and spread of the tad mutants in mice and normal biofilm formation in vitro demonstrate that the predicted Flp pilus is not required for the ability of Mtb to form community associations in culture or to infect in this mouse model.

DISCUSSION

For the most part, how Mtb-encoded adhesins contribute to virulence has remained a mystery. One of the only adhesins that has been demonstrated to have a role in Mtb pathogenesis is the heparin-binding haemagglutinin adhesion protein (HBHA), which recognizes receptors on epithelial cells (Pethe et al., 2000). HBHA was shown to be dispensable for colonization of mouse lungs and binding to phagocytic cells like macrophages, but was important for epithelial cell interactions and extrapulmonary spread of Mtb in a mouse model of infection (Pethe et al., 2001). It has recently been suggested that one or more predicted mycobacterial pili may play a role in Mtb adhesion to host tissue and virulence. In the case of MTP, this suggestion has been primarily supported by in vitro biofilm, attachment and invasion assays (Alteri et al., 2007; Ramsugit et al., 2013, 2016; Ramsugit & Pillay, 2014). Despite the uncertain contribution of MTP to virulence, these studies have spurred other groups to study MTP and MTP has been included as important for Mtb adherence in reviews on the subject (Govender et al., 2014; Hosseini et al., 2014; Ramsugit & Pillay, 2015). In addition to MTP, a putative pilus encoded by the widely conserved tad locus has also been suggested to be expressed and important in virulence (Alteri, 2005; Govender et al., 2014). However, to the best of our knowledge, the actual contribution of these factors to Mtb virulence had not been assessed before our study.

In this work, we found that MTP can contribute to in vitro pellicle biofilm formation in a strain-specific manner (Fig. 2). Mtb HN878 (Fig. 2) and V9124 (Ramsugit et al., 2013) strains require MTP for normal biofilm formation. The Mtb Erdman strain does not require MTP to form biofilms, but pellicle biofilm formation is more robust in an Erdman strain constitutively expressing MTP (Fig. 2). This enhanced pellicle in the Δmtp+ strain leads to increased tolerance to INH (Fig. 3). Despite previous studies suggesting that MTP may be a better adhesin than HBHA (Ramsugit et al., 2016), we found that the enhanced pellicle in the Δmtp+ strain does not translate to enhanced infection and spread in mice, nor does the deletion of mtp attenuate the bacteria during infection (Figs 4 and S3). In addition, we show that the tad locus genes are not required for Mtb Erdman virulence in C57Bl/6 mice, and at certain time points during infection the Δmtp and ΔtadC-flp strains actually had higher bacterial titres in infected lungs (Figs 4, 5 and S3).

While further work is required to understand why these deletion strains are at times more virulent, one could propose that the host immune system may target these molecules, so a decrease in antigen may actually be advantageous. In support of this, infection of C3HeB/FeJ mice with Erdman WT, Δmtp or Δmtp+ resulted in varying levels of histopathology at 56 d.p.i. (Fig. 4c–q), where Δmtp+ elicited the fewest number of encapsulated lesions and Δmtp elicited the most. However, it is not clear whether the difference in histopathology is due to differences in bacterial burden at 56 d.p.i. or due to differences in MTP expression. For instance, loss of mtp expression could lead to the formation of a higher number of encapsulated lesions where the bacteria replicate at high numbers extracellularly, contributing to the higher bacterial burden. Alternatively, loss of mtp expression could lead to a growth advantage and higher bacterial burden, which then leads to the formation of more encapsulated lesions. Whichever may be the case, different levels of mtp expression affect lesion architecture at 56 d.p.i., but MTP is not required for Mtb to survive in mice.

There are also multiple steps in human Mtb infection that are not mirrored in mice, including dystrophic mineralization (Driver et al., 2012), pulmonary cavitation (Wong &
Fig. 5. Effects of disruption of the tad locus on Mtb physiology and virulence. (a) Genetic organization of the tad operon maintained in the Mtb genome. Annotated functions of the tad genes are listed in the table. Regions selected for deletion are marked with black bars and labelled with the name of the deletion strain above the bar. (b) Southern blot analysis of tad deletion mutants. Left: for ΔtadAB, mutant and WT strain genomic DNA were digested with SacI, resulting in a WT 6.0 kb band or a 1.5 kb ΔtadAB band. Right: digestion with NheI yields a 1.4 kb WT band or a 4.4 kb ΔtadC/flp band. (c) Pellicle biofilm formation of tad mutants in 96-well plates. (d) Bacterial titres in the lungs (left) or spleen (right) of C57Bl/6 mice infected by the aerosol route with Erdman WT (black filled circles) or ΔtadC/flp (grey filled circles). Erdman WT and ΔtadC/flp infections were performed twice with three mice per time point per experiment; one experiment is shown. (e) Bacterial titres in the lungs (left) or spleen (right) of C57Bl/6 mice infected by the aerosol route with Erdman WT (black filled circles) or ΔtadAB (grey filled circles). The ΔtadAB infection was performed once with three mice per time point. Statistical significance between two groups was determined by Student’s t-test; *P<0.05.
The question of how Mtb adheres to different host tissues in vivo still stands. Mtb encodes many other adhesins and suggested adhesins (as reviewed in Govender et al., 2014), but the effects of most of these proteins are yet to be determined in vivo. This large number of predicted adhesins also raises the question of redundancy, which could confound analyses of single mutations. In addition, with about half of the Mtb genome encoding hypothetical proteins that have not been studied (Mazandu & Mulder, 2012), it cannot be ruled out that Mtb encodes novel factors important for host cell adherence that have yet to be identified. In conclusion, although neither MTP nor Flp are required for infection and spread in mice, the questions of how Mtb adheres and how that adherence relates to pathogenesis are important and remain wide open for future study.

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REFERENCES


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**Figure 5—figure supplement 1.** VWM-load-dependent strengthening of 1:2 CFS between high-α and β oscillations is observed in largely in sensorimotor, but also in attentional brain systems. Low-frequency (LF, left) and high-frequency (HF, right) CFS hubs and their connections for significant positive correlations with VWM memory load (Load condition) for CFS between high-α and β frequencies at ratio 1:2 (as in Figure 5, all illustration details as in Figure 4a). DOI: 10.7554/eLife.13451.020