

Pili contribute to biofilm formation in vitro in *Mycobacterium tuberculosis*

Saiyur Ramsugit · Sinenhlanhla Guma · Balakrishna Pillay · Paras Jain · Michelle H. Larsen · Siva Danaviah · Manormoney Pillay

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Abstract Organized bacterial communities, or biofilms, provide an important reservoir for persistent cells that are inaccessible or tolerant to antibiotics. Curli pili are cell-surface structures produced by certain bacteria and have been implicated in biofilm formation in these species. In order to determine whether these structures, which were suggested to be encoded by the *Rv3312A* (*mtp*) gene, have a similar role in *Mycobacterium tuberculosis*, we generated a Δmtp mutant and a *mtp*-complemented strain of a clinical isolate of *M. tuberculosis* and analyzed these strains for their ability to produce pili in comparison to the wild-type strain. Phenotypic analysis by transmission electron microscopy proved the essentiality of *mtp* for piliation in *M. tuberculosis*. We then compared biofilm formation of the derived strains in detergent-free Sauton's media. Biofilm mass was quantified

spectrophotometrically using crystal violet. Furthermore, we examined *mtp* gene expression by quantitative real-time PCR in wild-type cells grown under biofilm versus planktonic growth conditions. We found a 68.4 % reduction in biofilm mass in the mutant compared to the wild-type strain ($P = 0.002$). Complementation of the mutant resulted in a restoration of the wild-type biofilm phenotype ($P = 0.022$). We, however, found no significant difference between *mtp* expression in cells of the biofilm to those growing planktonically. Our findings highlight a crucial, but non-specific, role of pili in the biofilm lifestyle of *M. tuberculosis* and indicate that they may represent an important target for the development of therapeutics to attenuate biofilm formation, thereby potentially reducing persistence.

S. Ramsugit · M. Pillay (✉)
Medical Microbiology and Infection Control,
School of Laboratory Medicine and Medical Sciences,
College of Health Sciences, University of KwaZulu-Natal,
1st floor Doris Duke Medical Research Institute,
Congella, Private Bag 7, Durban 4013, South Africa
e-mail: pillayc@ukzn.ac.za

S. Guma · B. Pillay
Microbiology, University of KwaZulu-Natal, Durban,
South Africa

P. Jain
Department of Microbiology and Immunology, Albert
Einstein College of Medicine, New York, NY, USA

P. Jain · M. H. Larsen
Howard Hughes Medical Institute,
Albert Einstein College of Medicine, New York,
NY, USA

M. H. Larsen
Department of Medicine, Albert Einstein College of
Medicine, New York, NY, USA

S. Danaviah
Africa Centre for Health and Population Studies,
University of KwaZulu-Natal, Durban, South Africa

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Introduction

Tuberculosis (TB) has been, and continues to be, one of the most widespread bacterial infections worldwide. The standard treatment for TB patients involves the use of multiple antibiotics for 6 months and the regimen for most patients with drug-resistant TB takes 20 months (WHO 2012). Whilst this rigorous approach to treatment has resulted in cure for close to 36 million people, 1.8 million people still die of the disease each year (Sasindran and Torrelles 2011). This is largely due to the prolonged therapy that results in patient non-compliance and challenges in delivery of long-term treatment in socioeconomically- and medically-poor countries with a high burden of disease (Spigelman and Ma 2004). Improved TB control is dependent on the development of newer drugs that can clear infection efficiently and within a shorter duration. This in-turn is dependent on a greater understanding into the mechanisms employed by the causative organism, *Mycobacterium tuberculosis*, to persist against the challenges of the competent host immune system and antibiotic pressure (Ojha and Hatfull 2012).

The most accepted hypothesis for the extensive TB treatment regimen is the existence of persisters, a subgroup of microorganisms that are phenotypically resistant to, or tolerant of, the commonly used antibiotics (Spigelman and Ma 2004). Microbial species are known to form biofilms (O'Toole et al. 2000). These self-assembled, organized, multicellular, and matrix-encapsulated structures are contributors to microbial persistence, promoting survival of its inhabitants in the face of environmental stresses (Costerton et al. 1999). Ojha et al. (2008) provided evidence that *M. tuberculosis* forms biofilms in vitro, which harbour drug-tolerant populations of cells that persist despite exposure to high levels of antibiotics. Their in vitro formation is common amongst isolates throughout the world suggesting that their formation is relevant to TB propagation or persistence (Pang et al. 2012).

Whilst the long-term persistence of *M. tuberculosis* in the presence of the host immune system and

chemotherapy bears a clear similarity to the chronic infections of biofilm-forming pathogens, it remains unclear whether this pathogen forms biofilms in the host (Ojha and Hatfull 2012). The non-tuberculous mycobacterial species, *Mycobacterium ulcerans* and *Mycobacterium avium*, do, however, colonize the host as biofilms (Carter et al. 2003; Marsollier et al. 2005). Even though *M. tuberculosis* may be restricted to the phagosome during early stages of infection, many of the bacteria likely experience an extracellular environment later on, when lesions contain liquefied caseum and when patients are highly infective (Ojha and Hatfull 2012). This is further exemplified by the observations that TB lesions, including open cavities, contain numerous extracellular bacilli growing in multicellular structures (Canetti 1955) and by the presence of surviving micro-colonies in infected guinea pigs after drug treatment, located around the acellular rim in the granulomas (Lenaerts et al. 2007).

Microbial community behaviour is complex and can involve many genetic loci, particularly those that encode extracellular factors that promote surface colonization or cell-to-cell contact (Barnhart and Chapman 2006). Several bacteria, including *Escherichia coli* and *Salmonella* spp., are known to produce extracellular fibres called curli pili that are important mediators in biofilm formation (Barnhart and Chapman 2006). Although it was previously believed that mycobacteria did not produce pili, Alteri et al. (2007) provided compelling evidence for the existence of *M. tuberculosis* curli-like pili (MTP). Fibre analysis identified the *Rv3312A* (*mtp*) gene product as a component of MTP. These researchers also provided initial genetic evidence for the role of *mtp* in piliation by generating Δmtp mutants of *M. tuberculosis* H37Rv and CDC1551 that lacked detectable MTP (Alteri et al. 2007).

In the present study, we sought to provide further genetic evidence for the requirement of *mtp* in pili production by generating Δmtp mutant and *mtp*-complemented strains of a *M. tuberculosis* clinical isolate; evaluating *mtp* gene expression in these strains by quantitative PCR; and assessing their ability to form pili by transmission electron microscopy. Furthermore, we aimed to determine whether these surface appendages contribute to *M. tuberculosis* biofilm formation in vitro and evaluate *mtp* gene expression of wild-type cells growing in biofilms versus planktonic conditions.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium tuberculosis V9124, a drug-susceptible clinical isolate of the F15/LAM4/KZN (KZN) family, isolated from the Tugela Ferry (KwaZulu-Natal, South Africa), together with its mutant and complemented strains, were propagated on solid Middlebrook 7H10 or 7H11 medium (Difco), containing 10 % (v/v) oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton–Dickinson) and 0.5 % (v/v) glycerol (Sigma) or for planktonic growth in liquid Middlebrook 7H9 medium (Difco), supplemented with 10 % (v/v) OADC, 0.5 % (v/v) glycerol, and 0.05 % (v/v) Tween 80 (Sigma). *E. coli* strains were cultured in Luria–Bertani (LB) broth or agar (Difco). The concentrations of antibiotics used were 150 µg ml⁻¹ hygromycin (Roche Applied Sciences) and 30 µg ml⁻¹ kanamycin (Sigma) for mycobacterial strains, and 150 µg ml⁻¹ hygromycin and 40 µg ml⁻¹ kanamycin for *E. coli*.

Bioinformatics of the *mtp* gene and its translation product

The *mtp* gene sequence and locus was obtained from the TubercuList website (Lew et al. 2011). The *mtp* gene sequence of the clinical isolate used in this study was confirmed by PCR amplification and sequencing (N. Naidoo, S. Ramsugit, and M. Pillay; submitted). Secondary structure and topology of the corresponding amino acid sequence were determined using the PredictProtein software (Rost et al. 2004). Signal peptide was predicted using SignalP 4.0 server (Petersen et al. 2011). Protein hydrophobicity was predicted using a Kyte–Doolittle Hydrophathy Plot (Kyte and Doolittle 1982).

Construction of *mtp* deletion mutant ($\Delta mtp::\gamma\delta sacBhyg\gamma\delta$)

An allelic exchange substrate (AES) designed to replace the *mtp* gene with a hygromycin-resistance (*hyg*^R)-*sacB* cassette was kindly donated by W. R. Jacobs, Jr., AECOM. This had been generated by PCR amplification of the sequences flanking the left and right regions of the *M. tuberculosis mtp* gene using the primer pairs Mtp-LL and Mtp-LR, and Mtp-RL and Mtp-RR, respectively

(Table 1). Subsequently, upstream and downstream arms were digested with BstAPI and Van91I, respectively, and cloned into Van91I-digested pYUB1471 vector arms. The plasmid pYUB1471 allows one step generation of AES and contains a *hyg*^R-*sacB* cassette, lambda phage *cos* sites, and a unique PacI site to clone into temperature-sensitive mycobacteriophage phAE159 shuttle phasmid. The resulting AES was digested with PacI and ligated with PacI-digested phAE159, in vitro packaged using a MaxPlax packaging extract (Epicentre Biotechnologies), and transduced in the *E. coli* HB101 strain (Invitrogen Life Technologies) for construction of shuttle phasmids. The donated phasmids were electroporated into *Mycobacterium smegmatis* mc²155 (kind gift from W. R. Jacobs, Jr., AECOM) and incubated at 30 °C for phage propagation (Bardarov et al. 2002). Specialized transduction of the *M. tuberculosis* V9124 wild-type strain was performed as described by Bardarov et al. (2002). A 188-bp region of the *mtp* gene was subsequently deleted and replaced with a $\gamma\delta res-sacB-hyg-\gamma\delta res$ cassette, and hygromycin was used for the selection of mutants.

PCR screening of deletion mutants

Genomic DNA of the putative deletion mutants was isolated using the NaCl-cetyl trimethylammonium bromide method (Ausubel et al. 1989). The 312-bp *mtp* gene was amplified using primer pairs Mtp-1F and Mtp-1R, to generate a 370-bp product, as well as Mtp-2F and Mtp-2R, to generate a 583-bp product (Table 1), to confirm the loss of the *mtp* gene in the mutant compared to the wild-type strain. The PCR mix contained: 1X buffer with 1.5 mM MgCl₂, 250 µM dNTPs, 0.2 µM of each primer, 2 U *Taq* polymerase (Roche Applied Sciences), and 100 ng DNA per 25 µl of reaction mixture. PCR amplification was performed under the following conditions: 95 °C for 1 min; 40 cycles of 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s; and a final cycle with an extension time of 5 min at 72 °C. All standard PCR amplifications were carried out on the GeneAmp PCR System 9700 (Applied Biosystems) and amplicons were resolved by electrophoresis in a 1.5 % (w/v) agarose (Seakem) gel, containing ethidium bromide, and visualized in a UV trans-illuminator (Syngene G-Box, Vacutec).

Replacement of the *mtp* gene by the *hyg*^R-*sacB* cassette was further confirmed by amplification of a 300-bp portion of the *hyg*^R cassette using 2 µM of

Table 1 Primers and probes used in this study

Name ^a	Purpose	Primer/probe sequence ^b	Product size (bp)
Mtp-LL	Upstream allelic exchange substrate primers	5'-TTTTTTTTGCATAAA <u>TTGGTCACGATT</u> CATGCCGGTGAG-3'	731
Mtp-LR		5'-TTTTTTTTGCATTTTCGCCGCCACTGTGAAT CAGGTGTGATGTCACCTGAGGTC TCTGTTGGGCTGGGACTGC-3'	
Mtp-RL	Downstream allelic exchange substrate primers	5'TTTTTTTCATAGATTGGGGCTGTGTAGCACTCCTACGAGTGTCTGGTCTCGTA GAAAGTCCCGTGTGACCGAT-3'	776
Mtp-RR		5'-TTTTTTTTCCATCTTTTGGAAATTCGGTCCCGTTGAAA-3'	
Mtp-IF	Amplification of region containing <i>mtp</i> gene	5'-GATCGCTTTCGTACCGTCAT-3'	370
Mtp-IR		5'-ACGCGTATTTCGCTGATGC-3'	
Mtp-2F	Amplification of region containing <i>mtp</i> gene	5'-CTCATGGGTACACAGCGAGTA-3'	583
Mtp-2R		5'-ATGACACAGGTTCCCTTCAAGC-3'	
Hyg-F	Amplification of region of <i>hyg</i> ^R cassette	5'-ACCCCCCATTCGGAGGTCCTT-3'	300
Hyg-R		5'-CCGGAAGCGTTGAGATGCA-3'	
INS-1	Amplification of MTB's insertion sequence	5'-CGTAGGGCATCGAGGTGGC-3'	245
INS-2	<i>IS6110</i>	5'-GCGTAGGGCTGGTGACAAA-3'	
SH-F	Generation of probe for Southern hybridization	5'-GCCGTCCGATGACAGAAAAT-3'	783
SH-R		5'-AACAAACGCACCGAATAGCCG-3'	
Mtp-261F	Amplification of <i>mtp</i> gene for insertion into pMV261 for complementation	5'-TTTTTAAAGATCTCATGTACCGGTTCCGGTG-3'	436
Mtp-261R		5'-TTTTTAAAGCTTGGCACCGGAGCGTAAATCTG-3'	
Mtp-AnyF	Primers and probe for detection of <i>mtp</i> expression in qPCR	5'-CCCAACTGGGATCCCTACAC-3'	73
Mtp-AnyR		5'-AGTCGGGCTGTGGTC-3'	
<i>mtp</i> probe		FAM-CTGCCATGACGACTTC-MGBNFQ	
16S-AnyF	Primers and probe for detection of 16S rRNA expression in qPCR	5'-GGGTGACGGCCCTACCAA-3'	54
16S-AnyR		5'-GCCGGACACCCCTCTCA-3'	
16S probe		FAM-CCGGCTACCCGTCGTC-MGBNFQ	

^a Forward primer listed above the reverse primer for each primer pair

^b Restriction sites are underlined

each primer Hyg-F and Hyg-R (Table 1), 1X buffer with 500 μ M MgCl₂, 200 μ M dNTPs, 2.5 U *Taq* polymerase, and 100 ng DNA per 50 μ l of reaction mixture. The thermal cycling conditions included: 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension of 7 min at 72 °C.

Southern hybridization analysis of the deletion mutant

A 783-bp fragment downstream of the *mtp* gene was PCR-amplified using primers SH-F and SH-R (Table 1). The amplification reaction mixture (25 μ l) contained: 1X buffer with 1.5 mM MgCl₂, 250 μ M dNTPs, 0.2 μ M of each primer, 1 U *Taq* polymerase, and 100 ng wild-type DNA. The cycling parameters were 95 °C for 10 min; followed by 40 cycles of 95 °C for 20 s, 50 °C for 1 min, and 72 °C for 1 min; and finally 5 min at 72 °C. The PCR product was labeled with Horse Radish Peroxidase (Amersham) and hybridized to *Nhe*I-digested wild-type and mutant genomic DNA and detected using enhanced chemiluminescence (Amersham; Millipore). The designed probe was expected to detect a 2,744-bp fragment for the wild-type and a 6,357-bp fragment for the deletion mutant.

Complementation of the Δ *mtp* strain

The *mtp* gene was cloned into the pMV261 vector (Stover et al. 1991) for complementation into the Δ *mtp* mutant strain as follows. A 436-bp fragment containing the *mtp* gene was amplified using PCR primers Mtp-261F and Mtp-261R (Table 1) that have *Bgl*II and *Hind*III restriction sites incorporated, respectively. The PCR mix contained: 1X buffer with 1.5 mM MgCl₂, 250 μ M dNTPs, 0.2 μ M of each primer, 1 U *Taq* polymerase, and 100 ng wild-type DNA per 25 μ l of total reaction volume. PCR amplification was performed under the following conditions: 95 °C for 10 min; 40 cycles of 95 °C for 20 s, 59 °C for 1 min, and 72 °C for 30 s; and a final cycle with an extension time of 5 min at 72 °C. The gel-purified (QIAquick Gel Extraction Kit, Qiagen) PCR product and vector were double-digested with restriction enzymes *Bgl*II and *Hind*III, and *Bam*HI and *Hind*III (Thermo Scientific), respectively. The digested PCR product was cloned downstream of the

mycobacterial *hsp60* promoter of the digested vector to generate pMV261::*mtp*. This ligation mixture was transformed into chemically-competent *E. coli* JM109 cells (kind gift from D. T. Claiborne, Emory University) and transformed cells selected by plating on LB agar plates containing kanamycin. Plasmid DNA was isolated from transformed colonies with the PureLink HQ Mini Plasmid Purification Kit (Invitrogen Life Technologies) and screened for the 436-bp *mtp* insert by PCR. Electrocompetent cells of the Δ *mtp* mutant strain were prepared and electrotransformed with the identified pMV261::*mtp* construct (Larsen et al. 2007). Transformants were selected on kanamycin-containing agar plates.

PCR screening and sequencing of *mtp*-complemented strain

Plasmid DNA was isolated from transformed *M. tuberculosis* colonies (Larsen et al. 2007) and the 436-bp sequence was once again amplified by PCR for confirmatory purposes. To ensure that no mutations were produced during amplification, and thereby confirming the integrity of the construct, PCR products were sequenced (Inqaba Biotec, Pretoria, South Africa) using the same primer set, and Chromas Pro and Bioedit software were used to analyze the chromatograms and perform multiple sequence alignment.

IS6110, a *M. tuberculosis* complex-specific insertion sequence element, was amplified using primers INS-1 and INS-2 (Table 1) (van Soolingen et al. 1991) to confirm that the derived Δ *mtp* mutant and *mtp*-complemented strains were *M. tuberculosis*, which was also confirmed by Ziehl-Neelsen staining.

RNA extraction and quantitative real-time PCR (qPCR)

In order to provide further evidence for the generation of the Δ *mtp* mutant and *mtp*-complemented strains, *mtp* gene expression in the wild-type, mutant, and complemented strains were analysed by qPCR. After the cultures were grown aerobically on 7H11 agar plates for 7 weeks at 37 °C, total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies), precipitated with isopropanol overnight, resuspended in Ultra-pure DNase/RNase-free water (Biolone), and the concentration and purity were determined using a

NanoDrop (Thermo Scientific). Total RNA (200 ng) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Roche Applied Sciences) according to the manufacturer's protocol. Real-time PCR analysis was carried out on the CFX96 Real-Time PCR Detection System (Bio-Rad). Five microlitres of the cDNA reaction was used in a 25 μ l reaction, containing 1X *TaqMan* Universal PCR Master Mix (Applied Biosystems), 4 mM primer Mtp-AnyF, 4 mM primer Mtp-AnyR, and 2.5 U μ l⁻¹ *mtp* probe (Table 1) with a 5'-FAM and 3'-minor groove binder (MGB) non-fluorescent quencher (NFQ). The amplification protocol involved 95 °C for 10 min and 50 cycles of: 95 °C for 15 s and 60 °C for 1 min. Expression was normalized against the expression of 16S rRNA housekeeping gene, using primers 16S-AnyF and 16S-AnyR and the 16S probe (Table 1). Each reaction was repeated with three independent RNA samples. Negative/no template controls were included in each experiment. Normalized expression (using the $\Delta\Delta$ Cq method) was determined using the CFX Manager Software and results were expressed as fold change in expression of the target gene in the mutant and complemented strains relative to the wild-type expression.

Transmission electron microscopy (TEM)

The wild-type, mutant, and complemented strains that were grown on 7H11 agar for 9 weeks were fixed in 4 % paraformaldehyde overnight at room temperature. After heat-killing at 80 °C for 1 h, a drop of bacterial suspension was placed onto 200 square mesh copper grids (Agar Scientific) and removed after 5 min. The samples were negatively-stained with 2 % aqueous uranyl acetate for 5 min and rinsed with a drop of sterile water. The dried samples were viewed using a JEOL 1010 transmission electron microscope at an accelerating voltage of 100 kV.

Biofilm growth conditions

Using a modification of published protocols (Ojha et al. 2008); the wild-type, mutant, and complemented strains were cultured for biofilm growth in 6-well polystyrene plates (Porvair). Cells from 7H9 medium-aerated cultures (absorbance at 600 nm [A_{600}] = 1) were diluted 1:100 (v/v) in Sauton's medium without

detergent and 2 ml added to each well in triplicate. The plates were wrapped twice in Parafilm and incubated at 37 °C in 5 % CO₂ for 5 weeks without shaking. Serial dilutions of the bacterial inocula were plated in triplicate onto 7H11 plates to confirm that similar numbers of viable cells were included for all three strains in the assay. Plates were assessed visually after 5 weeks for biofilm formation.

Quantification of biofilm mass

Crystal violet staining for the quantification of biofilm mass was conducted by modifying previously described methods (Carter et al. 2003; Pang et al. 2012). Medium was aspirated by pipetting. The remaining biofilm mass was dried in a biosafety cabinet and incubated with 0.5 ml of 1 % crystal violet solution for 10 min. The wells were washed thrice with water and dried again. Two and a half millilitres of 95 % ethanol was added to each well for 10 min and the absorbance of the resulting biofilm cell-associated dye was measured at A_{600} on a spectrophotometer (Biochrom WPA Lightwave II, Labotec), using identically-treated planktonically-grown cultures to blank each sample.

qPCR analysis

Total RNA from 5 week old planktonic and biofilm wild-type cultures was extracted and qPCR used to evaluate *mtp* expression during biofilm growth relative to that during planktonic growth, as described above.

Results and discussion

Organization of the *mtp* gene locus

A schematic illustrating the chromosomal organization of the *mtp* (*Rv3312A*) gene in the wild-type and in a *mtp* deletion mutant strain is shown in Fig. 1a. The *mtp* gene is located between genes involved in intermediary metabolism (*add*, *deoA*, and *cdd*) and a conserved mycobacterial gene of unknown function (*Rv3312c*), an organization notably different to the operons of pili biogenesis genes observed in other curli piliated bacteria. In *E. coli* and *Salmonella* spp.,

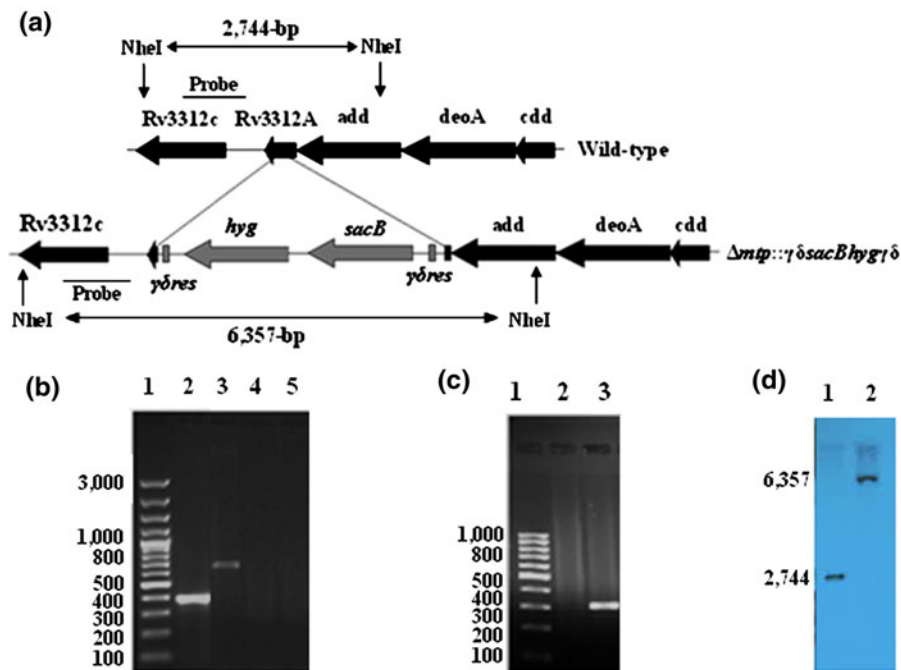


Fig. 1 Construction of the *mtp* deletion mutant by allelic replacement and its confirmation. **a** A schematic illustrating the chromosomal organization of the wild-type (upper) and the Δmtp mutant (lower) strains, in which the *mtp* gene has been disrupted by a *hyg^R-sacB* cassette, and expected sizes following restriction with *NheI* and Southern hybridization. **(b, c)** Confirmation of the mutant by PCR. In **b** the *mtp* gene was amplified using primers Mtp-1F and Mtp-1R, as well as Mtp-2F and

Mtp-2R, yielding products of 370-bp and 583-bp in the wild-type (Lanes 2 and 3), but absent in the mutant (Lanes 4 and 5). In **c** the hygromycin-resistance cassette was detected using primers Hyg-F and Hyg-R, yielding a 300-bp product in the mutant (Lane 3), that is absent in the wild-type (Lane 2). **d** Southern blot showing digested genomic DNA of the wild-type (Lane 1) and mutant (Lane 2), probed downstream of the *mtp* gene. The location of the probe is also illustrated in the schematic in **(a)**

curli pili operons include the *csgBA* and *csgDEFG* (Hammar et al. 1995) and the *agfBA* and *agfDEFG* operons (Collinson et al. 1996), respectively, with both species requiring at least six proteins for the production of curli. However, pili genes are not always arranged in clusters and there is a possibility that the MTP biogenesis genes are located distantly on the chromosome (Alteri 2005).

The absence of any sortase-encoding genes in *M. tuberculosis*, that are normally required for pili assembly in Gram-positive species (Paterson and Mitchell 2004); the lack of a β -sheet structure typically seen in curlins and amyloid fibres (Table 2); a general limited knowledge on curli pili in Gram-positive organisms; coupled with the complexity of the cell wall architecture of *M. tuberculosis*, point to a unique system of pili biogenesis in this organism (Alteri et al. 2007).

Generation of the *M. tuberculosis* $\Delta mtp::\gamma\delta sacB hyg\gamma\delta$ (Δmtp) mutant and complemented strain

In order to confirm the role of the *mtp* gene in pili production, a precise deletion mutant of *mtp* was generated by deleting 188-bp of the 312-bp *mtp* sequence using specialized transduction (Bardarov et al. 2002) (Fig. 1a). This deletion of about 60 % of the sequence of the *mtp* gene is expected to suffice in hindering functioning of the translation product, as protein sequence analysis using a combination of bioinformatics tools showed that the deleted region includes important functional domains (Table 2). This includes the transmembrane region that is necessary for spanning the protein across a biological membrane; the signal peptide region necessary for secretion of the protein; 51.85 % of the amino acids

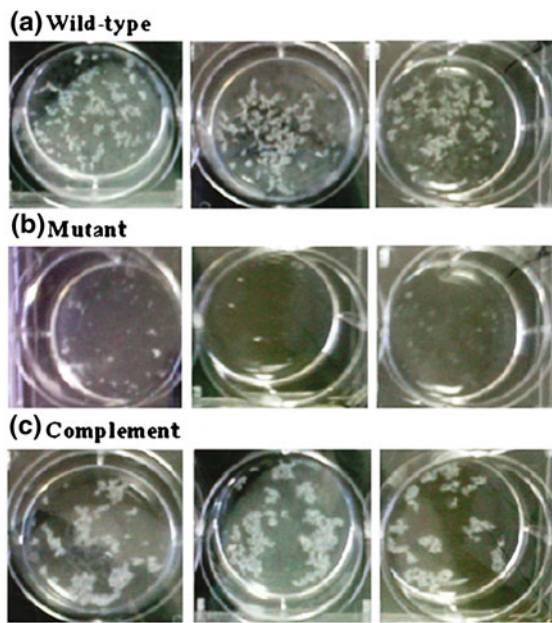


Fig. 3 The requirement of *M. tuberculosis* pili (MTP) in biofilm formation. *M. tuberculosis* V9124 wild-type (a), Δmtp mutant (b), and *mtp*-complemented (c) strains were inoculated into Sauton's media in 6-well plates and incubated for 5 weeks at 37 °C and 5 % CO₂ without shaking

Evidence that the *mtp* gene is involved in pili production

To demonstrate the phenotypic consequence of the *mtp* gene knockout and the subsequently-derived complemented strain, transmission electron microscopy was employed to directly view negatively-stained pili. The wild-type strain was shown to be piliated (Fig. 2a). Only 16 of the 20 individual wild-type cells produced pili-like structures, possibly due to the absence of signals and environmental cues required for pili expression during in vitro growth (Alteri 2005).

Expression was, however, much higher than the 10–20 and 10–15 % piliated bacteria reported by Alteri et al. (2007) and Velayati et al. (2012), respectively. The higher percentage piliation seen in this study highlights the strain- and condition-specific expression of curli, and is most likely largely due to the older cultures used here (9 weeks, as opposed to 2–3 weeks), as nutrient limitation has been shown to stimulate curli gene expression in organisms such as *Salmonella typhimurium* (Gerstel and Romling 2001). This occurrence was confirmed by wild-type *mtp* gene expression analysis by qPCR of a 5 week versus

15 week culture in which *mtp* expression was 6.35-fold higher in the older culture. This observation indicates that *mtp* are possible genetic determinants involved in the long-term dormancy and persistence of *M. tuberculosis*.

The pili structures identified by electron microscopy were hair-like and aggregated to form a network/mesh of fibres that extended out of the cell surface to varying degrees. They also seemed to facilitate inter-bacterial adherence between these organisms, suggesting that *mtp* are possibly genetic loci involved in cell aggregation and biofilm formation.

Analysis of the Δmtp mutant strain demonstrated an absence of surface appendages. Every cell examined ($n = 30$) lacked pili (Fig. 2b). TEM of the complemented strains demonstrated that piliation was clearly restored (Fig. 2c). Copious amounts of pili fibres were expressed by every cell examined ($n = 20$). These results support those of the transcript analysis and, taken together, confirm that the *mtp* gene is required for pili formation in *M. tuberculosis*.

Pili contribute to *M. tuberculosis* biofilm formation

To determine whether MTP contribute to biofilm formation, we assessed the ability of the wild-type, Δmtp mutant, and *mtp*-complemented strains to form biofilms in vitro. The wild-type strain produced a biofilm phenotype (Fig. 3a). Cells were aggregated to each other forming several sediments and a pellicle at the liquid-air interface. In the absence of MTP, in the pili-deficient mutant strain, biofilm formation was clearly defective. There was no liquid surface growth and minimal attachment to the container surface in comparison to the wild-type (Fig. 3b). Colony forming unit (CFU) determinations confirmed that a similar number of bacterial cells was included for the wild-type and mutant biofilm assays (8.3×10^7 and 7.8×10^7 CFU's per ml, respectively). This indicated that the defect in biofilm formation in the mutant wells was not due to inoculation of low viability mutant inoculums. Furthermore, subsequent growth of the biofilm-grown mutant cells under planktonic conditions resulted in restored growth, similar to the wild-type cells in a planktonic state, indicating that the lack of biofilm formation in the mutant was not due to growth defects or a loss of viability.

Crystal violet staining for biofilm mass determination showed a 68.43 % reduction in biofilm mass in the

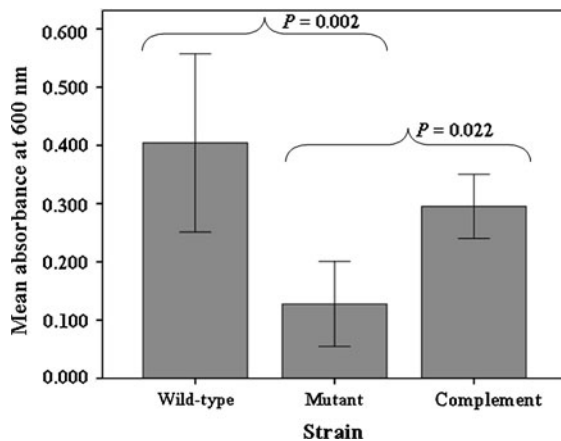


Fig. 4 Crystal violet quantification of biofilm mass of the wild-type, mutant, and complemented strains. Results are expressed as mean absorbance values at 600 nm, derived from triplicate samples. Standard deviations are also shown. The experimental data were analyzed by one-way analysis of variance (ANOVA) using SPSS 21.0 statistical software; $F(2,6) = 21.969$, $P < 0.05$

mutant compared to the parental strain ($P = 0.002$) (Fig. 4). Complementation resulted in a restoration of the wild-type biofilm phenotype ($P = 0.022$) (Figs. 3c, 4). The sediments seen in the pili over-expressing *mtp*-complemented strain were much larger than in the wild-type, suggesting the role of pili in facilitating aggregation and biofilm maturation.

To determine whether pili expression is enhanced during biofilm growth, we assessed *mtp* gene expression in the V9124 wild-type strain cultured under planktonic and biofilm growth conditions by qPCR. It was, however, found that *mtp* expression in cells of the biofilm were similar to those growing planktonically.

Whilst *mtp* expression is not up-regulated or specific to the biofilm lifestyle, the results of this study, nevertheless, indicate that pili are important contributors to the community lifestyle of *M. tuberculosis*. Their role most likely would be to facilitate surface attachment and cell-to-cell contact, thus contributing to the structural integrity of the biofilm. Alteri (2005) showed that statically grown *M. tuberculosis* H37Ra adhered to glass coverslips and each other using pilus-like structures. Their possible role in aggregation to other bacterial cells was previously noted by Velayati et al. (2012) using atomic force microscopy of clinical isolates of *M. tuberculosis*. This cellular aggregation may be attributed to the highly hydrophobic nature of the MTP protein (Alteri 2005).

Gao et al. (2004) suggested the role of *mtp* in another complex *M. tuberculosis* aggregative phenotype known as cording, a phenomenon associated with virulence in a variety of models (Middlebrook et al. 1947). Using microarray technology, they showed that the *mtp* gene was consistently expressed at higher levels in H37Rv, a virulent, cording strain of *M. tuberculosis*, compared with H37Ra, an avirulent, non-cording strain derived from the same original patient isolate.

The finding by Alteri et al. (2007) that MTP are expressed in vivo and bind strongly to the eukaryotic extracellular matrix protein laminin supports the notion that pili could also be actively-engaged in surface attachment and biofilm formation in vivo (Islam et al. 2012).

Concluding remarks

Since biofilms are important reservoirs for the development of drug-tolerant persistent organisms, the identification of the role of MTP in biofilm formation in this study indicates that they may represent an important target for the development of new, more effective drugs against TB. These drugs, termed curlicides (Cegelski et al. 2009), that block the formation of curli, would disrupt biofilm formation, and when used in combination with current anti-TB drugs may enable a much shorter and more effective treatment regimen. Further studies are underway to identify the role of MTP in other aspects of TB pathogenesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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