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Abstract: **Introduction** *Mycobacterium tuberculosis* curli pili (MTP) are novel, potential TB diagnostic biomarkers, possessing important virulence attributes, unique to the *M. tuberculosis* complex (MTBC). The production of high quality recombinant transmembrane and secretory proteins that can serve as biomarkers may be challenging due to their secretion attributes. For example, the signal peptide of MTP governed by the classical secretion pathway may hinder the purification of the protein in *E. coli* systems. In this study, the secretion characteristics of MTP were determined and the cloning, expression and purification of MTP was attempted in *E.coli.*

Materials and methods A fragment of MTP unique to MTBC was cloned into pet101 and pGEX-6P-1 vectors. The clones were confirmed by nucleotide sequencing and expression of the protein was attempted at IPTG concentrations ranging from 0.1mM to 1mM and at temperatures between 25 °C to 37 °C. The pGEX-6P-1/*mtp* clone expressed protein was purified, yielding a MTP-GST fusion protein and a free GST band that were analysed by LC/MS mass spectrometry. Inclusion body preparation attempted from the pet101/*mtp* clone yielded two bands at 10 kDa and below 10 kDa, both of which were analysed by LC/MS mass spectrometry. Transcription activity of both the clones was interrogated by real time PCR on the cDNA derived from the induced clones at various time points after induction with IPTG. The signal peptide and protein secretion characteristics of the MTP protein were determined by bioinformatics analysis of the amino acid sequence using publically available software.

Results The truncated MTP fragments were successfully cloned in both the vectors as confirmed by nucleotide sequencing. Expression of the pGEX-6P-1/*mtp* clone using 0.5 mM IPTG at 27 °C demonstrated the presence of the expected fragment at approximately 35 kDa. This was confirmed by Western Blotting using anti-GST antibodies. However, the purification of MTP in adequate quantities as a pure protein fraction was unsuccessful. Mass spectrometry did not detect any fragments belonging to MTP, but only those of *E.coli* membrane proteins for the pet101/*mtp* clone and fragments of the GST tag in the case of the pGEX-6P-1/*mtp* clone. The bioinformatics secretion analyses of MTP predicted a strong Sec regulated secretion pathway and the absence of non-classical "mycobacterial specific" secretion.

Discussion *M. tuberculosis* membrane and secretory proteins often contain signal peptides. In this study, excluding the signal peptide region and using a GST tag greatly enhanced the expression of the protein in the soluble fraction. However, purification of the MTP peptide remained problematic due to a lower available peptide concentration resulting from the lower molecular weight, in the purified fraction compared to the GST tag. Alternately, the predicted Sec regulated secretion pathway may play a role in the inhibition of MTP overexpression in *E.coli*. Thus, alternatives to *E. coli* expression systems or more efficient purification strategies are required for the acquisition of high quality *M. tuberculosis* antigens.

Introduction

Novel biomarkers for diagnostic, therapeutic and preventative strategies are pertinent to tuberculosis (TB) control [1]. *Mycobacterium tuberculosis* curli pilus (MTP), a cell surface appendage and adhesin, has recently been highlighted as an important virulence factor and biomarker for the design of such interventions [2]. The potential accuracy of MTP for use in a diagnostic test was also recently demonstrated, as this adhesin is unique to *M. tuberculosis* complex (MTBC) pathogens [2,3]. Consequently, the availability of the MTP antigen is essential for studies on TB diagnostics, pathogenesis and immunological response to *M. tuberculosis* infection and disease.

The *mtp* gene in its entirety has previously been cloned and expressed in *Escherichia coli* [4]. While the recombinant protein was not visible in Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), it was detectable by western blot, reacting with rabbit anti-MTP antibodies generated using a synthetic peptide. These antibodies, confirmed with recombinant MTP were utilized in ImmunoGold electron microscopy to detect purified pili fibres and pili involved in adhesion and invasion assays. However, the recombinant MTP protein was not purified from *E. coli* total protein in that study.

Previously reported difficulties in visualising purified MTP by Coomassie blue and silver staining were ascribed to its resistance to chemical treatments such as acid hydrolysis and denaturation with formic acid, urea, Triton X-100, and enzymatic digestion with Proteinase K, cellulose and lysozyme [5]. Pili were observed either in the stacking gel or as bands that were too large to be considered as pilin proteins that are usually observed as 6-25kDa fragments [5]. Thus, it is envisaged that recombinant MTP protein production will alleviate the difficulties encountered in the isolation and characterisation of purified MTP into subunits.

E. coli cloning and expression systems are commonly used for proteins from diverse bacteria. This is largely due to the rapid and confluent growth of *E.coli* on inexpensive media, its well known genetics and the availability of a range of cloning vectors, host strains and soluble fusion tags [5]. Several *M. tuberculosis* proteins have been expressed in *E. coli* including the 38-kDa, Mtb81 antigens [6], ESAT6, CFP10, MTC28, 14-kDa antigen [7], Shikimate Kinase and 5-Enolpyruvylshikimate 3 Phosphate Synthase Enzymes [8], among others. Up to 50% of *M. tuberculosis* transmembrane proteins cloned into *E.coli* compatible vectors were shown to be successfully expressed [9], but interestingly, only 25% of these clones were overexpressed, resulting in higher protein yields. Whilst this study proved that *E. coli* compatible vectors may be a good first attempt for expression of some

M. tuberculosis transmembrane proteins [9], other researchers have shown that >50% of *M. tuberculosis* proteins cannot be expressed in this host system [5,10].

The inadequate expression of *M. tuberculosis* protein in *E. coli* may be due to the significant differences in the G-C composition of *E.coli* (50%) and *M. tuberculosis* (65-70%), resulting in differences in codon usage between the mRNA of these 2 microbes [11,12]. Usually a small number of rare codons do not severely depress protein expression [11]. However, the presence of numerous rare codons may lead to low protein yield. These problems can be overcome by enriching the cells with the essential rare tRNA by inserting the tRNA genes into multiple copy plasmids, thereby increasing the copy number available in the cell [11]. Commercially available *E. coli* strains are also available for this purpose.

Another major reason for inefficient expression of *M. tuberculosis* proteins is that Mycobacteria are known to post-translationally ADP-ribosylate proteins [13], whereas post-translational modifications are not common in *E. coli* proteins [5,13]. In addition, proteins expressed in *E. coli* are sometimes located in inclusion bodies, a consequence of high-level protein production in the *E. coli* cytoplasm [5]. However, the use of highly soluble protein partners such as Glutathione-S-transferase (GST) and induction at lower temperatures usually negates this problem. Monitoring of transcription levels of genes during induction in clones and confirming mRNA production can elucidate possible post-translational modifications or shunting of the protein into inclusion bodies [5].

The "classical secretion pathway" is governed by the Sec and Tat signal peptides made up of a structured three component motif found at the N-terminal of the proteins [14]. The Tat motif is similar to the Sec motif except that the former contains a Tat motif contains a twin-arginine consensus sequence. Unfolded and folded proteins are secreted through the membrane using the Sec- and Tat-dependent secretion pathways, respectively. These systems transport proteins across the inner membrane in Gram-negative bacteria, whilst additional secretion systems operate in the outer membrane. Signal peptides play a role in these secretion systems. Little is known about pilin secretion and assembly mechanisms through the outer membrane of the "diderm mycolate" bacteria [15]. Thus, it is not surprising that very little information can be found on the secretion of the curli pilin subunit across the cell wall of mycobacteria.

The purpose of this study was to study the pilin secretion pathway using bioinformatics and to attempt the cloning and expression of a unique fragment of MTP. In the present study, the *E. coli* cloning and expression system was used in an attempt to generate and purify the truncated MTP protein

as these are inexpensive and robust systems for the isolation of high concentrations of *M. tuberculosis* proteins. The truncated *mtp* gene was successfully cloned into the pet101 and pGEX-6P-1 vectors. However, the apparent lack of overexpression of truncated MTP was insufficient for proper protein purification and subsequent detection using mass spectrometry. Protein secretion characteristics of the pilin subunit determined the presence of a signal peptide governed by the classical secretion pathway and which may play a role in shunting the pilin subunit out of the cell in the presence of the signal peptide. Our findings indicate that secreted and membrane protein signal peptides and secretion pathways need to be ascertained before recombinant protein expression

is attempted. Moreover, a more efficient system for the expression of mycobacterial transmembrane and extracellular proteins is required.

Materials and Methods

Transmembrane, rare codon and secretory protein analysis of the pilin subunit

The secondary structure and secretory properties of the entire MTP protein were determined using bioinformatics tools (Table 1). Rare codons were identified using the nucleotide and amino acid sequences of the *mtp* gene [12].

Table 1. The Bioinformatics software used to analyse the MTP amino acid sequence with the details of the specific analyses.

Tool	Details	website	Citation
Kyte-Doolittle	Hydrophobicity - ProtScale on the Expasy server	web.expasy.org/protscale/	(38)
TMMTOP	Transmembrane domain	http://www.cbs.dtu.dk/services/TMHMM/	
Phobius	Combined transmembrane topology and signal peptide predictor showing N, H and C regions	http://phobius.sbc.su.se/	(39)
SignalP	Signal peptide cleavage prediction	http://www.cbs.dtu.dk/services/SignalP/	(40)
PRED-TAT	Determine classical or non-classical secretion pathway	http://www.compgen.org/tools/PRED-TAT/	(41)
TatP	Tat motifs and associated signal peptide	http://www.cbs.dtu.dk/services/TatP/	(42)
TBPRED	*M. tuberculosis* specific prediction tool for subcellular localisation prediction	www.imtech.res.in/raghava/tbpred/	(43)

Plasmids and bacterial strains

Cloning and expression of the *mtp* gene were performed in the pet101 (Invitrogen) and the pGEX-6P-1 (GE Healthcare) cloning vectors (Supplementary figure 1). The pet101 vector contained a T7 promoter, *lac* promoter, T7 termination site, Ampicillin resistance gene and C-terminal 6× His tag (~4 kDa). The pGEX-6P-1 vector contained a *tac* promoter, *lac* promoter, Ampicillin resistance gene, multiple cloning site containing 9 restriction enzyme sequences and an N-terminal GST tag (26kDa) containing a PreScission protease cleavage site. Cloning and plasmid propagation were carried out in One Shot TOP10 Chemically Competent *E.coli* cells

(Invitrogen). Expression was performed in either the BL21Star Chemically Competent Cells (Invitrogen) or the *E. coli* Rosetta 2(DE3) pLysS™ cells (Novagen) that contained pRARE plasmids containing a chloramphenicol resistance gene.

Construct Design and Cloning

The cloning strategies including the *mtp* gene-specific primers used to generate the PCR product and the DNA string used for ligation into the pet101 and pGEX-6P-1

vectors respectively, are depicted in Figure 1. Inserts of 64 (7.04 kDa) and 33 (3.63 kDa) amino acids, were cloned into the pet101 vector and pGEX-6P-1 vectors respectively.

The H37Rv strain was used as a reference genome for the design of cloning constructs.

The Rv3312A gene sequence was obtained from Tuberculist: http://genolist.pasteur.fr/TubercuList/genome.cgi.

with shaking at 200 rpm for 1 hr. Volumes of 10 µl, 50 µl and 100 µl of the ligation reaction mix were plated onto Luria-Bertani (LB) agar plates containing 100 µg/mL Ampicillin, and incubated at 37 ºC, overnight. Single colonies from the plates were grown in 5 mL LB broth containing 100 µg/mL Ampicillin at 37 ºC with shaking (200 rpm). In addition, colony PCR was performed to screen for the insert and vector-specific primers flanking the T7 region (Table 2). The region was sequenced to confirm the gene was in frame with the 6× His tag. The plasmids from the positive
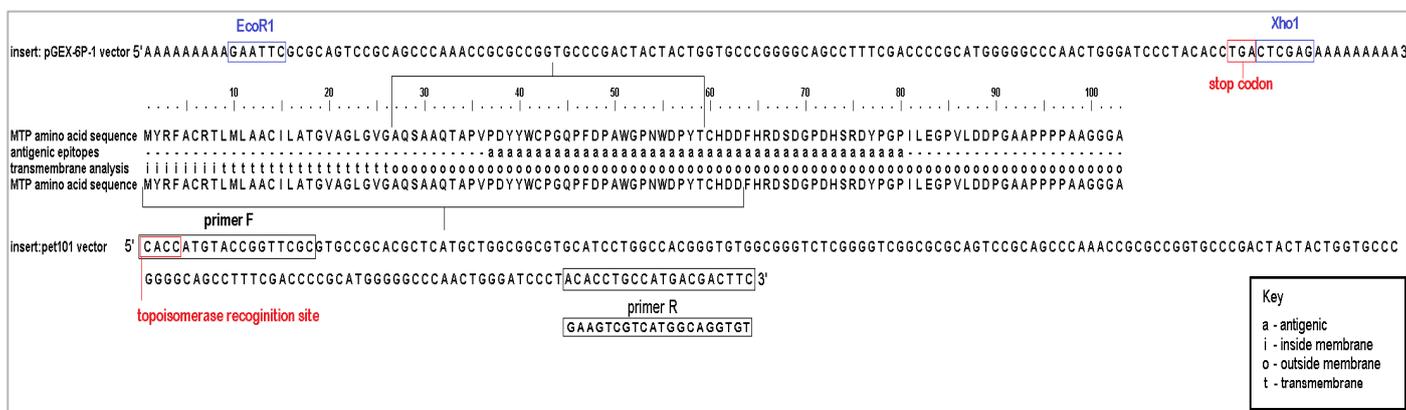

Figure 1 The pet101 and pGEX-6P-1 cloning strategies that were designed based on the Rv3312A (*mtp* gene) amino acid sequence, nucleotide sequence and the protein secondary structure prediction (ProteinPredict).

Generation of pet101/mtp clones

The insert fragment for the pet101 vector was generated by PCR in a 25 µl reaction, containing 1× Taq buffer, 1.5mM MgCl$_2$, 0.2 µM forward and reverse primers (Table 2), 0.2 mM dNTPs, 1 U Platinum® *Taq* Polymerase, 20 ng/µl *M. tuberculosis* H37Rv genomic DNA, and nuclease free water. The cycling conditions were: 94 ºC for 1 min; 35 cycles of 94 ºC for 30 s, 50 ºC for 1 min and 72 ºC for 1 min and a final extension of 72 ºC for 5 min. Following size confirmation in an agarose gel and purification using the QIAquick Gel Extraction kit (Qiagen), the PCR product was confirmed by Sanger Sequencing (Inqaba Biotech) to confirm amplification of the correct gene.

Ligation was performed in a 1:2 ratio of vector:insert, by combining 1 µl of salt solution™, 2 µl of sterile water, 1 µl of the pET101 vector (20 ng/µl) and 2 µl of PCR product (20 ng/µl) and incubated for 20 min at room temperature, according to the Champion™ pET Directional TOPO kit (Thermo Fisher Scientific). Thereafter, 6 µl of the ligation reaction were transferred into two separate vials of One Shot® TOP10 Chemically Competent *E. coli* cells, and incubated on ice for 30 mins. This was followed by incubation in a 42 ºC water bath for 30 s, and immediately placed back on ice. Two hundred and fifty microliters of SOC medium were added to the vial and incubated at 37 ºC

colonies were isolated using the Probond Plasmid Purification Kit (Invitrogen) and transformed into the appropriate *E. coli* expression strains before expression.

Generation of pGEX-6P-1/mtp clones

A double stranded DNA string (90 bp fragment of the *mtp* gene) was synthesized (Life Technologies) and cloned into the pGEX-6P-1 vector as follows. The pGEX vector and *mtp* gene insert (1 ug each) were double digested with Fastdigest EcoR1 (1U) and Xho1 (2U) (ThermoScientific) in a 20 µl reaction. The restricted DNA samples were gel extracted using the QIAquick Gel extraction kit (Qiagen).

Ligation was accomplished with a 1:3 vector:insert ratio in a 20 µl reaction containing 2.42 µl pGEX-6P-1 vector DNA, 2.8 µl insert DNA, 4 µl ligation buffer, 1 µl T4 DNA ligase (Invitrogen) and 9.78 µl nuclease-free water. After incubation at room temperature for 5 min, 2 µl of ligation reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* cells as described above.

Positive clones were confirmed by colony PCR using primers shown in Table 2, and Sanger sequencing as described above. The positive colonies were treated the same as the pet101/*mtp* clones.

Table 2 Primers used in this study

Primer	Sequence	Description
mtpF	5' CACCATGTACCGGTTCGC 3'	Generated the insert for the pet clone
mtpR	5' GAAGTCGTCATGGCAGGTGT 3'	
T7 promoter	5 TAATACGACTCACTATAGGG 3'	Used for screening colonies and sequencing after
T7 reverse	5' TAGTTATTGCTCAGCGGTGG 3'	cloning and transformation of the pet clone
pGEX 5'	5' GGGCTGGCAAGCCACGTTTGGTG 3'	Used for screening colonies and sequencing after
pGEX 3' Sequencing Primers	5' CCGGGAGCTGCATGTGTCAGAGG 3'	cloning and transformation of the pgex clone
mtp_F	5' CAGTCCGCAGCCCAAACC 3',	Target *mtp* gene primer used for Real time PCR
mtp_R	5' CGAGTCAGGTGTAGGGATCC 3'	transcript analysis of the *mtp* gene
16S_F	5' CCTACGGGAGGCAGCAGT 3',	Housekeeping gene primers used for Real time PCR
16S_R	5' CGTTTACGGCGTGGACTAC 3'	transcript analysis of the *mtp* gene

Protein Expression

Expression of the pet101/*mtp* and pGEX-6P-1/*mtp* clones was optimised using concentrations of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.1mM, 0.3mM, 0.5mM, 0.7mM and 1mM, and at temperatures of 25°C, 28°C, 30°C and 37°C (Table 3). Protein expression was attempted in both *E. coli* BL21 (DE3) and *E. coli* Rosetta 2(DE3) pLysS™ cells (Novagen), and separated on either 12 or 16% denaturing SDS-PAGE while the tags were detected by western blotting using anti-His antibody (donated by Dr Nonhlanhla Nene, National Bioproducts Institute) for the pet101/*mtp* clone and anti-GST antibody (donated by Dr Nonhlanhla Nene, National Bioproducts Institute) for the pGEX-6P-1/*mtp* clone.

Protein Purification of the pGEX-6P-1/*mtp* clone using glutathione agarose

The sample lysate of the expressed clone was prepared by mixing the protein extract with Equilibration/Wash Buffer to a total volume of 20 mL and transferred to a 10 mL Glutathione Agarose column (Pierce) that had been equilibrated with 100 mL of the same buffer. The column was washed several times until the absorbance of the flow-through fraction at 280 nm approached baseline (0 mg/ml). Thereafter, the GST-tagged protein was eluted in 1mL

fractions with 20 mL of Elution Buffer and monitored by measuring the A_{280}. The Amicon Pro Purification System (Merck) with a 10 kDa molecular weight cut off filter was used to remove glutathione and concentrate the purified protein for downstream applications. PreScission protease (GE Healthcare) was used to cleave the GST tag from the peptide after purification.

Inclusion body preparations

The insoluble pellet fractions from induced samples processed after sonication were screened for expressed peptide by boiling in 5×SDS-PAGE Laemmli buffer and separated by electrophoresis in SDS-PAGE in order to determine whether the cloned protein may be expressed in inclusion bodies.

Further analysis of the inclusion bodies was performed by inducing the pet101/*mtp* clone at 1 mM IPTG at 30 °C and the pGEX-6P-1/*mtp* clone at 0.5 mM IPTG at 27 °C. Induced cells were centrifuged at 6000 rpm for 20 min, and the pellet re-suspended in 3ml Buffer A (50mM Tris-HCl, pH8, 5 mM EDTA and 10mM NaCl) per gram of cells. Thereafter, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 16 µl lysozyme (50 mg/ml) per gram of cells were added and incubated at 37 °C for 30 min. The solution was sonicated to reduce viscosity and centrifuged at 18 000 rpm for 30 min. The remaining

pellet was re-suspended in 3 ml Buffer B (20 mM Na_2HPO_4, pH 7.2, 20 mM NaCl, 5 mM EDTA and 25 % sucrose) per gram of cells. PMSF was added to a final concentration of 1mM followed by 10 µl Triton X-100 per ml of solution. Following centrifugation at 20 000 rpm for 20 min, the remaining pellet of inclusion bodies was dissolved in 8 mM

Urea with heating at 37 °C. The urea was dialyzed in 4 L of 50 mM Tris-HCl buffer at pH 8.5 using a 3.5 kDa molecular weight cut off dialysis tubing (Sigma) for 2 days, with a buffer replacement every 8 hrs. Proteins were concentrated by dialysis with PEG 20 000.

Table 3 The pilot protein expression conditions of the pet101/*mtp* and pGEX-6P-1/*mtp* clones at various temperatures and IPTG concentrations as assessed on SDS-PAGE gels

Temp (°C)/ IPTG (mM)	BL21(DE3) *E.coli* cells					Rosetta2(DE3)pLysS *E.coli* cells				
	0.1	0.3	0.5	0.7	1	0.1	0.3	0.5	0.7	1
pet101/*mtp* clone										
25	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-
pGEX-6P-1/*mtp* clone										
25	-	-	-	-	-	+	+	++	+	+
28	-	-	-	-	-	+	+	++	+	+
30	-	-	-	-	-	+	+	+	+	+
37	-	-	-	-	-	+	+	+	+	+

Mass spectrometric analysis of the putative recombinant peptides

Bands obtained from SDS-PAGE of inclusion body preparations of the pet101/*mtp* clone overnight expression (10 kDa and <10 kDa), as well as from the pGEX-6P-1/*mtp* Glutathione agarose (Pierce) purified clone were excised and analysed by mass spectrometry (Central Analytical Facilities, Proteomics Laboratory, Stellenbosch University).

Analysis of mRNA levels during expression in the pet101/*mtp* and pGEX-6P-1/*mtp* clones

Due to the difficulty in visualising MTP fibres by Coomassie and silver staining on acrylamide gels demonstrated by Alteri *et al,* 2005, transcript analysis of the pet101/*mtp* and pGEX-6P-1/*mtp* clones was performed to ascertain whether the pilin gene was transcribed upon induction of expression.

Overnight cultures of cells freshly transformed with either the pet101/*mtp* clone or pGEX-6P-1/*mtp* clone were grown in 20 mL LB medium containing Ampicillin (100 µg/mL) at 37 °C in a shaking incubator. Cells at an OD_{600} of 0.5 were induced with 0.5 mM IPTG. Prior to IPTG addition, and at intervals of 5 min, 10 min, 15 min, 20 min, 30 min, 1 hr, 2 hr, 3hr, 4 hr and 5 hr after induction, samples were pelleted, frozen with liquid nitrogen and immediately stored at -80 °C.

Total RNA was isolated using the GeneJET RNA Purification Kit (ThermoScientific) and treated with the RNase-Free DNase (ThermoScientific) according to the manufacturer's instructions. RNA quantity and quality was assessed using the NanoDrop 2000 Spectrophotometer (NanoDrop Technologies). Reverse Transcription of 2 µg total RNA was performed with the High Capacity cDNA Reverse Transcription Kit (Applied BioSystems) in accordance with the manufacturer's protocol.

Real time PCR was performed in triplicate in 20 µl reaction volumes containing cDNA corresponding to 50 ng RNA and

Jumpstart ReadyMix SYBR Green (Sigma), in a BioRad CFX96-Real-Time System. The primers used are listed in Table 2. Amplification conditions comprised an initial activation step of 15 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 58.3 °C for 30 s and 72 °C for 30 s. To ensure comparability between data for the same target obtained from different PCR runs, the PCR efficiency (E) of the *mtp* gene-specific and 16S rRNA primers was determined using a standard curve as detailed in the Pfaffl method [16]. An E of 1.6 and 1.8 for each gene respectively was obtained at an annealing temperature of 58.3 °C (Supplementary figure S2).

For the calculation of the relative changes in gene expression, the Pfaffl method was applied taking the amplification efficiencies into account [16]. The fold difference in gene expression after induction was calculated using the following equation [16]:

$$\text{ratio} = \frac{E_{mtp}^{\Delta Cp_{mtp}(\text{control}-\text{sample})}}{E_{16S}^{\Delta Cp_{16S}(\text{control}-\text{sample})}}$$

Results

Bioinformatics of the MTP amino acid sequence reveals rare codons, hydrophobic and hydrophilic regions and Sec-dependent pilin secretion

Analysis of rare codons showed 12 sites at which the tRNA of the E. coli differed from that of M. tuberculosis. Of these 12, four occurred in the pGEX-6P-1/mtp clone sequence and 5 in the pet101/mtp clone sequence.

The Kyte-DoLittle software revealed a strong hydrophobic region at the beginning of the protein sequence and a strongly hydrophilic region thereafter, becoming slightly hydrophobic from around 80 amino acids (Figure 2). This is suggestive of the putative folding of the pilin subunit into the pilus shaft.

TMMTOP database identified an alpha helix region in the predicted hydrophobic domain (Figure 2).

Phobius database predicted the typical N-terminal signal sequence from substrates of either the Sec or the Tat systems bearing the polar n-region with a positive net charge, the uncharged hydrophobic h-region and short polar c-region [14] (Figure 2).

Curiously, the SignalP server predicted a cleavage site between amino acids 22 and 23, which is within the c-region rather than after it and also within the hydrophobic region. A possibly more accurate cleavage site between amino acids 33 and 34 was predicted by TatP. However, the prediction output demonstrated the absence of a twin-arginine (Arg = R), otherwise known as a Tat motif. In addition, TatP cleavage site prediction also corresponded to the PRED-TAT prediction with the cleavage site closer to amino acid 33, which consequently also predicted a more likely Sec associated protein secretion.

The bioinformatics analysis of the MTP amino acid sequence suggests that the Sec-dependent pathway is used to carry unfolded pilin subunits across the inner membrane into the periplasmic space in M. tuberculosis. This is an interesting finding, as it proposes that the MTP pilin subunit secretion is affected through the classical pathway similar to the pili of other bacteria [17] rather than the non-classical pathway that is unique to M. tuberculosis. Thus, even though MTP differs from other bacterial pili in terms of nucleotide sequence and gene organisation properties (not in an operon and distant from other pilin assembly components [2] pilin secretion into the periplasmic space has most likely remained the same.

The position specific analyses of the MTP amino acid sequence on TBPred software has predicted a protein with a lipid anchor which is highly favoured for M. tuberculosis as the bacterium has a lipid rich cell wall (Figure 2). This prediction needs to be confirmed experimentally.

The pet101/mtp clone showed no expressed product in the soluble fraction of the E.coli lysate as no apparent bands could be visualized by SDS-PAGE at the expected size of 10kDa in the induced or uninduced samples (Figure 4A). However, Western blotting using anti-His tag antibodies showed very faint bands, barely visible on images taken (data
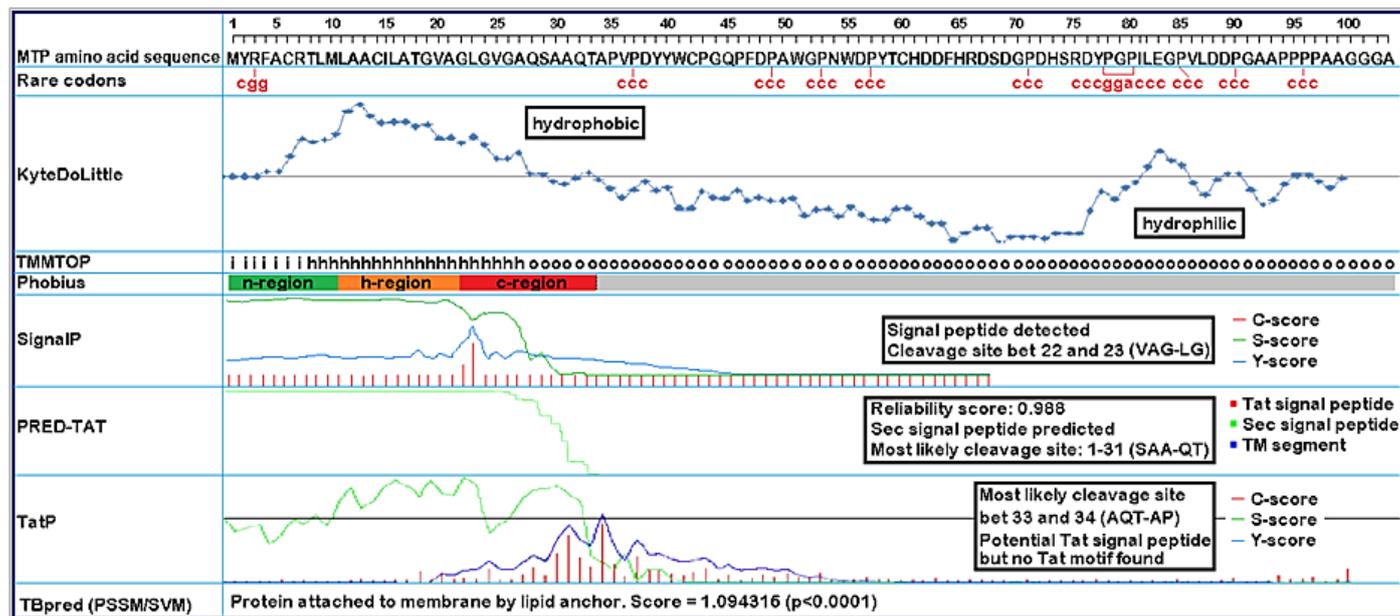
Figure 2 The amino acid sequence of the MTP protein showing the rare codons, the Kyte-DoLittle hydrophobicity and transmembrane analysis curves, with the scores calculated according to the properties of the amino acid sequence of MTP on the ProtScale server (web.expasy.org/protscale/), the Protein predict data with i = inside membrane, t = transmembrane and o = outside membrane, the TMMTOP data with i = inside membrane, h = alpha helix and o = outside membrane, Phobius data showing the n-region, h-region and c-region. The SignalP data with the predicted cleavage site between amino acids 22 and 23. The C score is the prediction for the cleavage site. The S score is the prediction for the amino acids that form a part of the signal peptide. The Y score is the average of the C-score and the S score, depicting a more accurate cleavage site prediction. PRED-TAT predicted a Sec signal peptide rather than a Tat signal peptide. TatP predicted a cleavage site between amino acids 33 and 34

Confirmation of the pet101/mtp and pGEX-6P-1/mtp clones

The pet101 and pGEX-6P-1 transformants were screened by colony PCR using the insert and Sanger sequencing of the vector-specific PCR products. In both cases, the cloning was successful and the inserts were both in frame with the respective fusion tags (Figure 3).

Expression of the proteins from the pet101/mtp clone, purification of expressed MTP from pGEX-6P-1/mtp clones and the acquisition of products sent for protein sequencing (mass spectrometry)

not shown). Neither E.coli BL21 (DE3) nor E. coli Rosetta 2(DE3) pLysS™ cells expressed detectable proteins in the pet101/mtp clone.

SDS-PAGE (Figure 4B) and western blotting using anti-GST antibodies demonstrated successful expression of pGEX6P-1/mtp clones transformed into E. coli Rosetta 2(DE3) pLysS™ cells. The expressed protein, present in the soluble fraction of the E. coli lysate, was purified using glutathione agarose and showed a strong reaction with anti-GST antibodies upon western blot analyses (Figure 5).

E. coli BL21 (DE3) cells transformed with either the pet101/mtp clone or the pGEX-6P-1/mtp clone failed to show any clearly visible expressed proteins on SDS-PAGE.

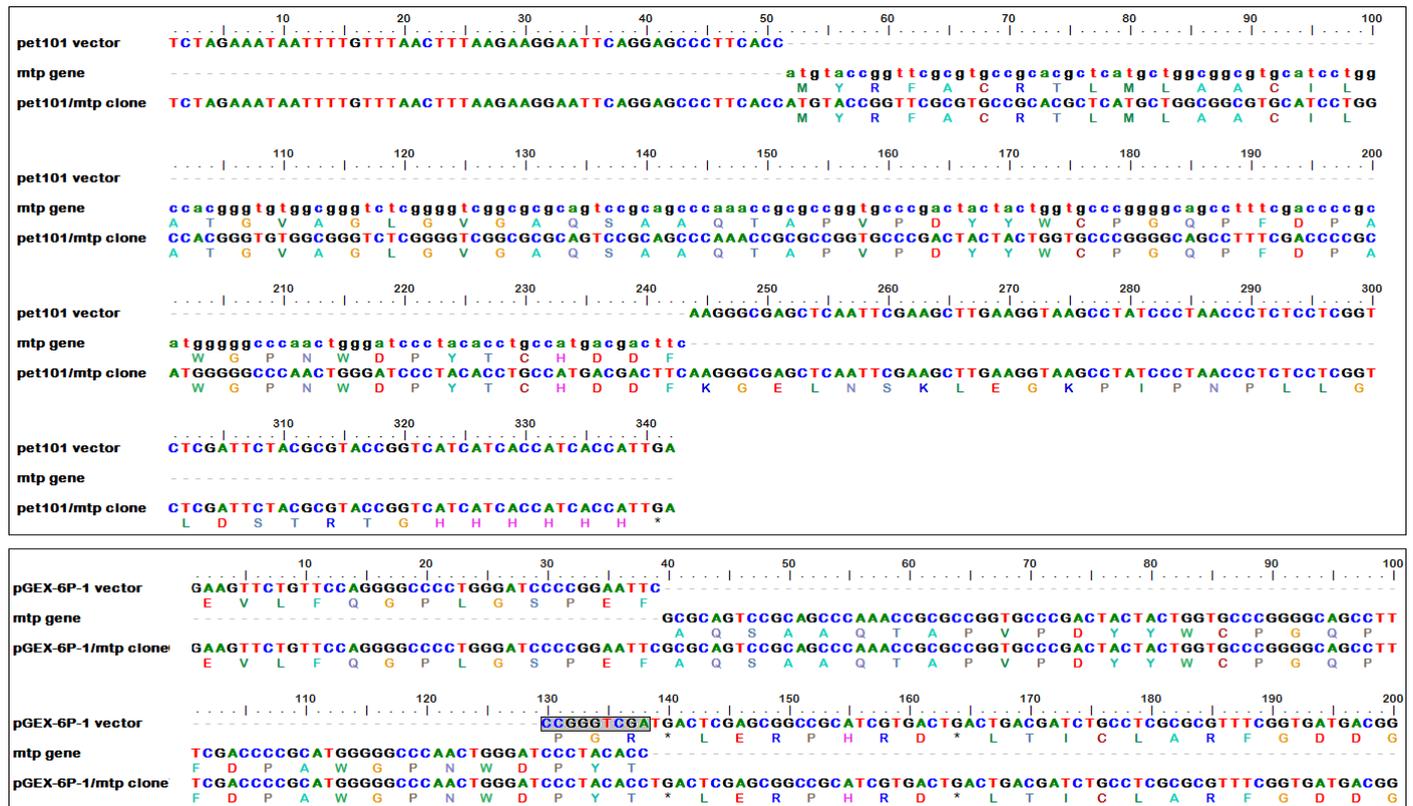on the SDS-PAGE gel as the peptide was too small to be seen.

Figure 3 Confirmation of pet101/*mtp* and pGEX-6P-1/*mtp* clones using vector-specific primers to generate PCR products and subsequent Sanger Sequencing. The shaded boxed sequence represents a part of the vector sequence that would have been cleaved by the restriction enzyme Xho1 during cloning of the insert into the vector.

The flow diagram (Figure 6) shows the steps that were taken in order to acquire putative MTP protein for protein sequencing by mass spectrometry.

Inclusion body preparation of the pet101/mtp clone revealed no overexpression of MTP protein (Figure 7A). Mass spectrometric analysis demonstrated that the 2 bands that were observed, at 10 kDa and below this, represented membrane proteins present in E.coli, and that no M. tuberculosis proteins were detected (Supplementary figure S3).

Protein sequencing of the pGEX-6P-1/mtp clone purified bands (Figure 7B) revealed fragments of the GST tag only, but not the MTP peptide upon BLAST analysis of the electrospray fragments to the M. tuberculosis H37Rv genome (Supplementary data S4). PreScission protease cleaved off the peptide from the GST as indicated by the loss of the larger band on SDS-PAGE and the presence of a remaining GST band. However, there was no indication of the peptide

Transcript analyses of the pet101/mtp clone and the pGEX-6P-1/mtp clone

The pet101/mtp clone showed a 28 fold less mtp gene expression than the pGEX-6P-1/mtp clone after 5 hours of induction. Overall, the transcription in the pGEX-6P-1/mtp clone occurred more rapidly than for the pet101/mtp clone. Thus, the data shows that the truncated mtp gene without the signal peptide is much more amenable to rapid, high level transcription as shown in the pGEX-6P-1 vector compared to the loner sequence that included the signal peptide in the pet101 vector.

Discussion

As the TB epidemic intensifies in developing countries, new diagnostic methods that are accurate, cost-effective and simple to implement are urgently required [18]. The typical diagnostic algorithm takes around 2 weeks, during which an individual can infect around 10-15 people before treatment initiation [19]. More sophisticated approaches in finding TB

biomarkers have been used in recent years in order to help pinpoint epitopes that are more sensitive and specific to M. tuberculosis [19]. Following identification of these markers, the antigens need to be accessible in large amounts for the generation of antibodies and for evaluation using patients' clinical samples.
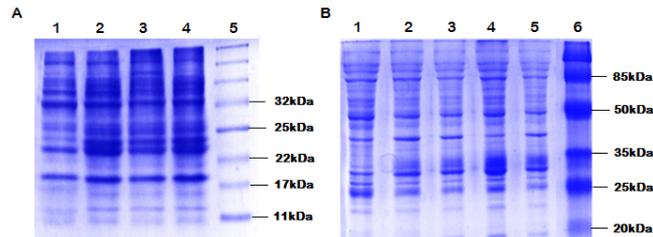

Figure 4. The SDS-PAGE gels of clones expressed at 28 °C: (A) 16% acrylamide gel: pet101/*mtp* clone Lane 1: uninduced sample, Lane 2: 0.3mM IPTG, Lane 3: 0.5mM IPTG, Lane 4: 1mM IPTG, Lane 5: Protein ladder (New England BioLabs Prestained protein ladder, cat number 7706), (B) 12 % acrylamide gel : pGEX-6P-1/*mtp* clone Lane 1: Uninduced sample, Lane 2: 0.1mM IPTG, Lane 3: 0.3mM IPTG, Lane 4: 0.5mM IPTG, Lane 5: 0.7mM IPTG, Lane 6: Protein ladder (Pierce Prestained Protein Marker: cat number 26612).

Although several promising antigens have been identified in previous studies, reproducible expression and purification of high-quality recombinant mycobacterial proteins is difficult and laborious [18]. In this study, recombinant DNA technology was used to attempt to generate high concentrations of pure MTP, an epitope that is highly specific to the M. tuberculosis complex [20]. Expression and purification of MTP was attempted using two vector systems containing different fusion tags (His tag and GST tag). However, the use of recombinant DNA technology proved inadequate for high
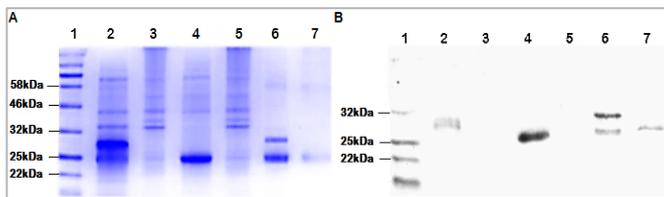

Figure 5 The expressed and purified MTP from the pGEX-6P-1/*mtp* clone. (A) SDS-PAGE and (B) Western blot, Lane1: Protein ladder (New England Biolabs, catalogue number: 7706), Lane 2: Induced pGEX-6P-1/*mtp* clone at 0.5mM IPTG and 28 °C. Lane 3: uninduced pGEX-6P-1/*mtp* clone, Lane 4: Induced pGEX-6P-1 vector control, Lane 5: uninduced pGEX-6P-1 vector control, Lane 6: Purified MTP, Lane 7: Purified pGEX-6P-1 vector control.

level purification of the truncated MTP protein.

In this study, rare codons were compensated for by the use of Rosetta[TM] cells (Novagen) containing "trare" plasmids. Our findings demonstrated that while Rosetta cells were capable of expressing MTP in the pGEX-6P-1/mtp clone, they were unable to enhance expression in the pet101/mtp clone. Since MTP was predicted to be a part of the transmembrane

proteins and is unable to be overexpressed in E. coli, this study may have revealed a similar challenge of inadequate expression of other transmembrane proteins in E. coli. Transmembrane proteins secreted via the classical secretion pathway may be shunted out of the cells in the presence of the signal peptide. This hypothesis needs to be further investigated using other transmembrane proteins with similar inadequate expression in E. coli, by exploring the signal peptides present in these proteins and monitoring transcription.

In a previous study, a 5 kDa product was detected by western blotting with anti-MTP antibodies on M. tuberculosis whole cell lysate, whereas a 14.5 kDa product, inclusive of 6X His tag (4 kDa), was detected by western blotting when expressed in E. coli [4]. Thus, it was postulated that E. coli lacked the post-translational modifications required to cleave the protein to 5 kDa. Alternatively, it could be hypothesized that a fraction of the protein may be exported from the cell without further assembly into pili on the cell surface, possibly due to the absence of the appropriate assembly factors or chaperones. Thus, most of the protein was likely secreted from the cell and therefore, not visible by SDS-PAGE in the expressed total protein lysate from E.coli but detectable in the more sensitive western blot.

The pet101/mtp clone that harboured the signal peptide was less successful at expressing detectable product in the SDS-PAGE and western blot than the pGEX-6P-1/mtp clone. Even though transcription of the pet101/mtp clone occurred in a time dependent manner at a much lower rate than the pGEX-6P-1/mtp clone, no apparent protein expression occurred even in the induced overnight culture, in either the soluble or insoluble fractions. Thus, it can be postulated that despite the slow progression in transcription, translation of the protein and Sec-associated cleavage of the protein that is observed in all bacterial species as part of the bacterial classical secretory pathway [14] may be responsible for the undetectable protein.

The absence of the N-terminal signal peptide recognition region and GST tag in the pGEX-6P-1/mtp clone may have resulted in protein being present in the soluble fraction of the E. coli lysate. However, the protein was not detected by sequencing using mass spectrometry, despite the discovery of several fragments of the GST tag. This inability to detect the peptide may have been due to its lower concentration following trypsin digestion from the GST tag. The peptide could not be visualised on SDS-PAGE after cleavage from the GST using PreScission Protease.
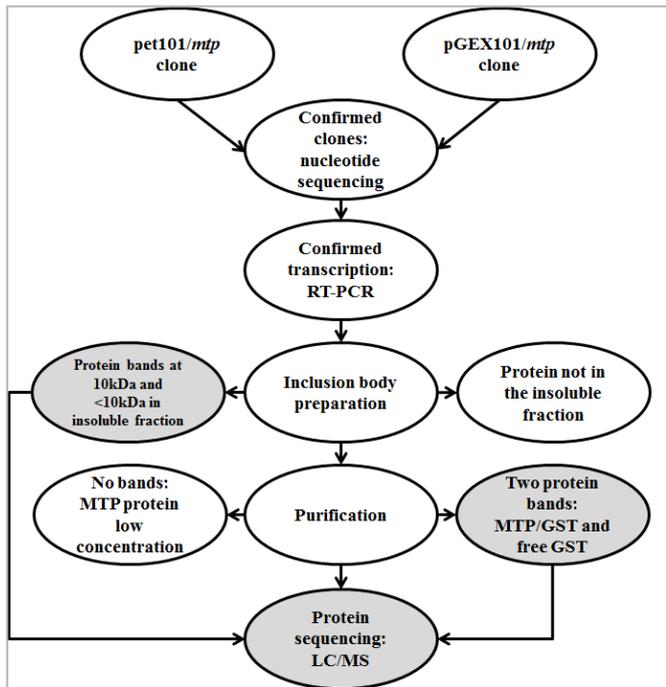
Figure 6 Process by which putative MTP products were obtained for protein sequencing and the results that were obtained at each step.

Unfolded pilin subunits are usually secreted out of the cell using the Sec dependent secretion system [17]. In Gram-positive bacteria, Sec secretion is often followed by pilin assembly mediated by assembly factors and chaperones depending on the type of pili being assembled. In Gram-negative bacteria, pili need to be secreted via a second membrane before assembly on the surface. Even though mycobacteria have been classified as a Gram-positive Actinomycete, the cell wall structure mimics that of Gram-negative bacteria. Thus, the pilin assembly of the Gram-negative curli pili may be more closely related to that of MTP. While MTP have been reported to harbour morphological and physicochemical similarities to the curli pili of Gram-negative bacteria [4], they differ in the nucleotide or amino acid sequence and genetic organisation [2].

Curli pili assembly are governed by two operons, one containing the pilin subunit CsgA and nucleator CsgB [21-23]. In the absence of CsgB, curlin assembly is attenuated as CsgB nucleates CsgA into a pilus fibre [23]. The second operon contains four other components required for pilin assembly. All the components of both operons are secreted through the Sec-dependent secretion pathway into the periplasm with the exception of CsgD, a transcriptional regulator of the csgBA operon. The pore through which CsgA and CsgB proteins travel through the outer membrane is made up of the CsgG protein. Salmonella curli pili have similar operons with assembly also governed by the nucleation pathway [24]. The components involved in pilin assembly of MTP are yet to be determined as the protein secretion through the outer membrane of mycobacteria has not been very well studied [15]. The secretion mechanisms of adhesins and other proteins on the outer membrane of mycobacteria would provide insight into the mechanisms through which these bacilli survive in the host cell. Nevertheless, the data in this study provides evidence that suggests that the pilin subunit is secreted through the inner membrane into the periplasm in much the same way as the pilin subunits of other bacteria.

A limitation of this study was that alternative approaches in order to express MTP were not attempted. An alternate approach to generate recombinant proteins specific to M. tuberculosis included the use of the yeast Pichia pastoris as a host instead of E. coli [25]. This approach has been shown to produce an end product at a yield of 0.5 g per litre of expressed culture using antigen bound to His tag. This is a significant yield when compared to membrane proteins expressed in E. coli with a maltose binding protein (MBP) tag that yields 10-90 mg antigen per litre of bacterial culture over a range of 22 M. tuberculosis proteins expressed [26]. In addition, the yeast derived antigen was better at detecting polyclonal antisera by western blot and antibodies in TB patient serum in ELISA [27].
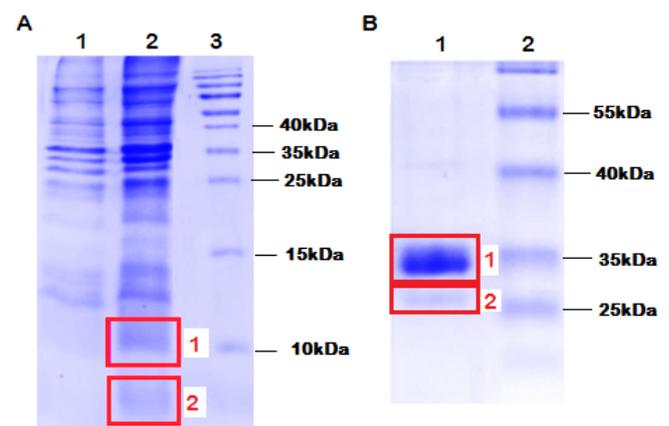

Figure 7 SDS-PAGE gels of (A) Expression products of inclusion body preparation of pet101/*mtp* clone induced with 1mM IPTG at 30 °C. Lane 1: uninduced inclusion body preparation, Lane 2: induced inclusion body preparation, Lane 3: PageRuler Prestained Protein ladder (cat number 26616). (B) Purification products of pGEX-6P-1/*mtp* clone induced with 0.5mM IPTG at 28 °C. Lane 1: purified protein, Lane 2: PageRuler Prestained Protein ladder (cat number 26616). The bands that were sequenced using mass spectrometry have been highlighted in red blocks.
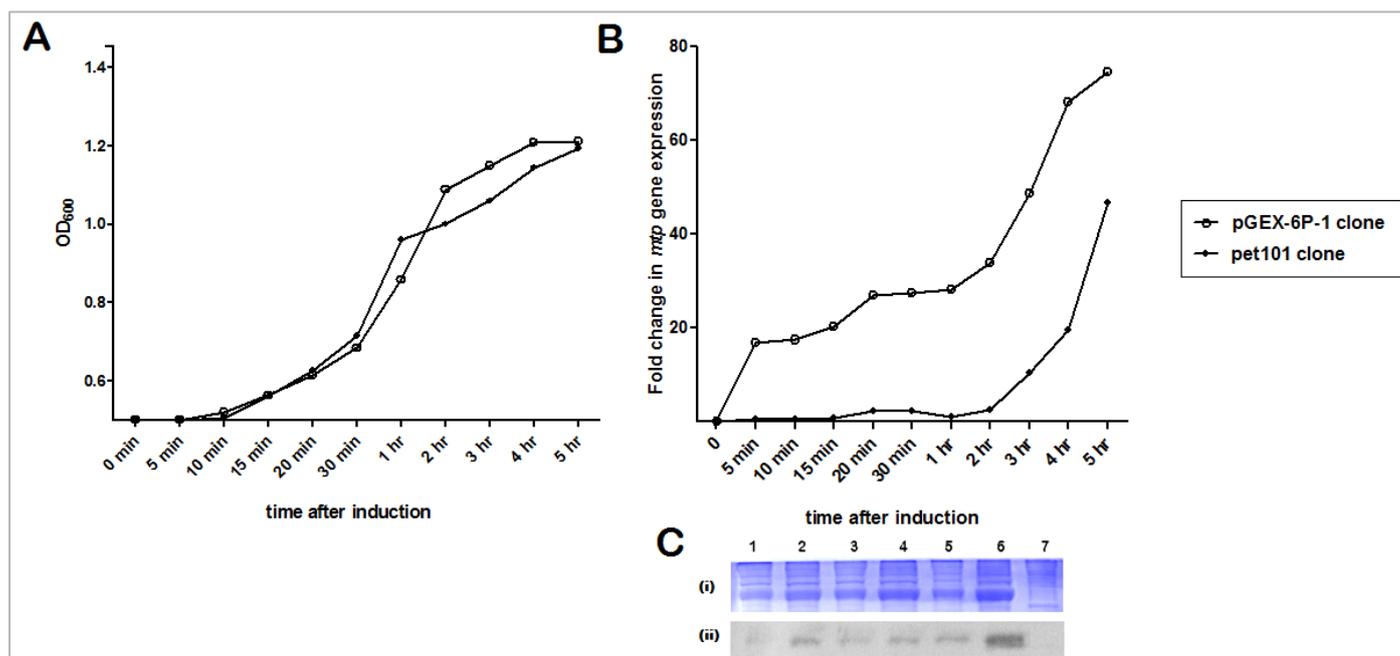
Figure 8 Analysis of (A) Growth curves of recombinant *E.coli* represented by OD_{600} readings at 5, 10, 15, 20 and 30 minutes and 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after induction with 0.5mM IPTG at OD_{600} = 0.5, (B) mRNA transcript analysis of clones represented by fold change at 5, 10, 15, 20 and 30 minutes and 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after induction with 0.5mM IPTG (C) (i) SDS-PAGE and (ii) western blot of the pGEX-6P-1/*mtp* clones, Lane 1: time point 0, Lane 2: 1 hour, Lane 3: 2 hours, Lane 4: 3 hours, Lane 5: 4 hours, Lane 6: 5 hours, Lane 7: uninduced control.

Mukherjee et al, 2003 reported the cloning of two or three copies of the same gene into a cloning vector in order to express the proteins as dimers or trimers [28]. This strategy was especially effective on proteins that were too small to be resolved on acrylamide gels and therefore, not easily detected upon expression. However, the achievement of a high success rate of these experiments involved several rounds of PCR using three or four different primer sets, and restriction and ligation of the inserts in order to produce an insert that could be fused with the vector. This requires a lot of technical skill and rigorous design of the insert generating strategy.

The use of water-soluble fusion tags are popular approaches and are usually effective for the expression of membrane proteins in particular [26]. In this study, the His-tag and GST fusion tags have been cloned alongside the MTP protein. The maltose binding protein (MBP) has not been cloned alongside MTP protein and this fusion tag has been used to successfully express M. tuberculosis transmembrane proteins [26]. Future studies include using MBP tags, with yeast as a host strain instead of E. coli or using an in-tube cell free protein expression system [29] rather than a host strain.

Synthetic peptides have also been used for the design of ELISAs for diagnostic purposes to overcome the problems associated with the low yield of recombinant M. tuberculosis proteins in E. coli [30-35]. Contamination associated with protein purification and from cloning vectors has been

reported to result in low yields of M. tuberculosis protein [36]. In addition, peptides were reported to be much more cost effective than expressed recombinant proteins [37] despite the advantage of multiple propagation and protein expression of clones.

The results of this study suggest that the curli pilin subunit is exported through the mycobacterial inner membrane through the Sec secretion pathway. Thus, we postulate that the pilin subunit is secreted through the inner membrane soon after translation by the Sec machinery and assembled through the outer membrane thereafter. If the pilin is assembled through the outer membrane, there are other yet undiscovered proteins involved in this process on the mycobacterial cell wall. The alpha helix transmembrane region may indicate that the N-terminal signal peptide region may double as a lipid anchor. The removal of the signal peptide containing region of the MTP greatly enhanced expression of the peptide in E.coli. This may indicate that the signal peptides of many other proteins may hinder the detection of expressed product when the protein is exported through the classical secretion pathway.

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