

Identification of *Mycobacterium tuberculosis* adherence-mediated components: a review of key methods to confirm adhesin function

Saiyur Ramsugit¹, Manormoney Pillay^{1*}

¹ Medical Microbiology and Infection Control, University of KwaZulu-Natal, Durban, South Africa

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ABSTRACT

Anti-adhesion therapy represents a potentially promising avenue for the treatment and prevention of tuberculosis in a post-antibiotic era. Adhesins are surface-exposed microbial structures or molecules that enable pathogenic organisms to adhere to host surfaces, a fundamental step towards host infection. Although several *Mycobacterium tuberculosis* adhesins have been identified, it is predicted that numerous additional adherence-mediated components contribute to the virulence and success of this pathogen. Significant further research to discern and characterize novel *M. tuberculosis* adhesins is, therefore, required to gain a holistic account of *M. tuberculosis* adhesion to the host. This would enable the identification of potential drug and vaccine targets for attenuating *M. tuberculosis* adherence and infectivity. Several methods have been successfully applied to the study and identification of *M. tuberculosis* adhesins. In this manuscript, we review these methods, which include adherence assays that utilize wild-type and gene knockout mutant strains, epitope masking and competitive inhibition analyses, extracellular matrix protein binding assays, microsphere adhesion assays, *M. tuberculosis* auto-aggregation assays, and *in silico* analyses.

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Introduction

Between 1990 and 2013, the global tuberculosis (TB) prevalence and mortality rates decreased by 41% and 45%, respectively (1). Despite the apparent progress in TB control, 9 million people developed TB and 1.5 million people succumbed to the disease in 2013, reiterating that this disease is still a severe global public health challenge (1). The World Health Organization's post-2015 global TB strategy aims to reduce the 2015 worldwide TB incidence and mortality rates by 90% and 95%, respectively, by the year 2035 (1). The achievement of these goals is dependent on the identification of novel biomarkers and targets for which new innovations for TB diagnosis, treatment, and prevention can be elucidated. These goals may be realized by intensifying scientific research into the interaction of the TB etiologic agent, *Mycobacterium tuberculosis*, with its human host.

A vital precursor to host colonization and the deploying of bacterial virulence factors, such as toxins and effector proteins, is the adhesion of the pathogen to

host cells. The initial binding to host cells occurs via weak and non-specific forces that are brought about by the physicochemical properties of the host surface. Thereafter, specific and transient receptor interactions enable a stronger anchorage of the pathogen to the host surface. Lastly, specific and high-affinity attachment is achieved by the interaction of bacterial adhesins with host receptors (2).

Adhesins are monomeric or filamentous cell-surface molecules or structures that facilitate bacterial attachment to host surfaces. This process is a pivotal precursor to bacterial invasion and colonization of the host, by limiting the clearance of the pathogen through shear stress and by activating key virulence factors that are associated with infection (3). Furthermore, bacterial adhesion to host cells results in a manipulation of host cell signalling, which promotes the spread of the pathogen and allows for the microbe to evade the host immune response (3). Adhesins also bind to receptors of the extracellular matrix (ECM), thereby activating signal transduction cascades in the host cell. These

*Corresponding author: Manormoney Pillay. Medical Microbiology and Infection Control, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa. Tel: +27-312604765; email: Pillayc@ukzn.ac.za

adhesins, termed microbial surface components recognizing adhesive matrix molecules (MSCRAMM), use receptors (such as integrins) to adhere to ECM molecules, including bone sialoprotein, collagens, elastin, fibronectin, glycosaminoglycans, laminin, thrombospondin, and vitronectin (4).

The ability of environmental bacteria to adhere to natural biopolymers (e.g., cellulose fibres) can be beneficial to these organisms, such as by providing access to nutrient-rich sources. In addition, the adhesion of bacteria to synthetic biomaterials (e.g., medical devices), such as those biomaterials that have been exposed to body fluids and contain adhered serum proteins, results in severe clinical consequences (4). Adhesin-mediated adhesion to host surfaces also enables biofilm formation (5), a multicellular lifestyle of bacteria that is associated with bacterial tolerance to antimicrobial agents, host defences, and other environmental challenges.

Due to their fundamental role in the host-pathogen interaction, bacteria have evolved a large variety of adherence-mediating components and strategies to enable their adhesion to the host. Pili are polymeric hair-like surface structures that contain an adhesin at its distal tip. They are the most researched class of bacterial adhesins, particularly in Gram-negative bacteria. Gram-positive organisms were also subsequently found to elaborate pili, and their function in adhesion and pathogenesis is being deciphered (6). A large arsenal of non-pilus adhesins have also been identified in bacteria. These adhesins are capable of interacting with host molecules, including transmembrane proteins and ECM components (7).

It is well-established that during infection, *M. tuberculosis* interacts with and enters host cells, including macrophages, epithelial cells, and dendritic cells. This process is crucial to the organism's survival, replication, and dissemination (8). However, the adhesins mediating these fundamental processes has been largely unexplored and underappreciated (9). Although several *M. tuberculosis* proteins have been documented to function in the attachment to host components (Table 1), computational tools have predicted an abundance of additional uncharacterized *M. tuberculosis* adhesins (Table 2).

A major challenge to the treatment of TB patients is the escalating rates of drug-resistant *M. tuberculosis* strains. A novel strategy for TB treatment and prevention involves targeting bacterial determinants of infection, rather than those of survival. This would incapacitate the pathogen and ensure a natural clearance of the bacilli, without imposing selective pressure and amplifying the number of drug-resistant forms of the bacterium. In addition, it would prevent the release of harmful

products (e.g., endotoxins) from lysed bacterial cells, which occurs with the use of bactericidal agents (2). However, due to a plethora of *M. tuberculosis* adhesion molecules, anti-adhesion TB therapeutics should target a combination of the major adhesins, possibly as an adjunct to the conventional TB therapeutics, to be effective. For adhesion inhibition to be a viable intervention for TB treatment and prevention, further clarity on the entire repertoire of *M. tuberculosis* adhesins is needed.

The purpose of this work is to review the major methods available for the identification of *M. tuberculosis* adhesins. These methods include adherence assays that utilize wild-type and gene knockout mutant strains, epitope masking and competitive inhibition analyses, ECM protein binding assays, microsphere adhesion assays, *M. tuberculosis* auto-aggregation assays, and *in silico* analyses.

Adherence assays that utilize wild-type and gene knockout mutant strains

Comparing the adhesion capacity of wild-type and gene knockout mutant strains is a widely used approach to identify genetic determinants of adhesion and infection. In this assay, wild-type and gene knockout mutant strains infect host cells (macrophages or epithelial cells) in microtitre plates. A multiplicity of infection of between 1:1 and 10:1 (bacilli to host cells) and an infection time of 1-4 hrs (usually 1 hr) is generally used. This is followed by washing to remove non-adherent bacilli and a lysis step (usually with 0.1% Triton X-100) to disrupt the host cells. Thereafter, serial dilutions of the cell lysates are plated onto agar plates to enumerate viable adherent bacterial cells, including those that have invaded the host cells. Since intracellular bacilli would have had to adhere prior to invading the host cells, the invaded bacteria also represent the total adhered population. However, one may distinguish between the adherent versus invaded fraction using the aminoglycoside protection assay (10) to quantify the invaded bacilli.

This colony-forming unit (CFU)-based adherence assay is advantageous in that it enables the cell association efficiency to be calculated from the total number of bacilli in the infection inocula. In addition, it is a simple and relatively low-cost method that requires no specialized equipment. This assay has been successfully used to identify the adhesin function of the Pro-Glu (PE) polymorphic GC-rich repetitive sequence (PGRS) protein encoded by *Rv1818c* (11), the heparin-binding hemagglutinin adhesin (HBHA) (12), and curli pili (MTP) (13, 14). In these studies, the deletion of the encoding genes (*Rv1818c*, *Rv0475*, and *Rv3312A*) resulted in a

significant reduction in *M. tuberculosis* adhesion to macrophages and/or epithelial cells.

This assay has also been used to measure the binding of *M. tuberculosis* to ECM proteins. Be *et al* (15) incubated wild-type and Δ *pknD* mutant *M. tuberculosis* strains with laminin for 1.5 hr. The wells were washed to remove unbound bacteria. The matrices were then lysed and plated onto agar plates for CFU assessment. A two-fold reduction in the adhesion to laminin was observed for the mutant, indicating that protein kinase D is a laminin-binding adhesin.

Epitope masking and competitive inhibition analyses

The deletion of essential (and even some non-essential) *M. tuberculosis* genes pose a technical challenge to the generation of gene knockout mutants. This prevents the utility of the previously described adherence assay to assess the possible adhesin function of the products that these genes encode. Epitope masking and competitive inhibition analyses are a useful alternate method in such cases. This strategy makes use of purified/recombinant proteins or antibodies against the purified/recombinant protein to block the binding of *M. tuberculosis* to the host components.

Hickey *et al* (16) used *M. tuberculosis* that was either untreated or treated with anti-Cpn60.2 antibodies to infect macrophages. Unattached bacteria were removed by washing and attached bacilli were identified by CFU counting and microscopy. The authors found that the *M. tuberculosis* strains with masked Cpn60.2 displayed a significant decrease in the adhesion to macrophages compared with the unmasked *M. tuberculosis*. The authors also used macrophages that were either untreated or treated with recombinant Cpn60.2 protein, prior to the addition of *M. tuberculosis*. The results indicated that *M. tuberculosis* displayed a decreased adhesion to Cpn60.2-treated macrophages compared with untreated macrophages. These findings implicate Cpn60.2 as a macrophage-associated adhesin.

A similar masking strategy was used by Abou-Zeid *et al* (17) to identify the BCG85 complex of *Mycobacterium bovis* BCG, which is also present in *M. tuberculosis* as the antigen 85 complex, as a fibronectin-binding adhesin. In this report, the authors showed that *M. bovis* BCG that was pre-incubated with anti-BCG85 antibodies demonstrated a diminished capacity to adhere to fibronectin compared with unmasked *M. bovis* BCG. They also found that fibronectin that was pre-incubated with purified BCG85A and BCG85B proteins inhibited the adhesion of *M. bovis* BCG to fibronectin.

ECM protein binding assays

In addition to their ability to bind to host cells, *M. tuberculosis* also adheres to ECM proteins, particularly collagens, fibronectin, and laminin. Therefore, numerous studies have identified *M. tuberculosis* adhesins based on their ability to bind to ECM proteins. Kumar *et al* (18) used an image-based enzyme-linked immunosorbent assay (ELISA) to demonstrate the binding of the recombinant Rv0309, Rv2599, and Rv3717 proteins to immobilized ECM proteins (fibronectin, laminin, and/or collagen). Detection of protein binding was achieved using horseradish peroxidase conjugated anti-6x histidine antibodies and ortho-phenylenediamine dihydrochloride.

Alteri *et al* (19) used flow cytometry to measure the binding of wild-type and MTP-deficient *M. tuberculosis* strains to ECM proteins, by the detection of anti-ECM antibody complexes, using Alexa Fluor conjugate. The binding of MTP to laminin was confirmed using a sandwich ELISA. In this experiment, the authors used varying concentrations of laminin to demonstrate that immobilized purified MTP protein bound to this ECM protein. Bound laminin was detected using anti-laminin antibodies and IgG peroxidase conjugate. Similarly, using flow cytometry and ELISA, Pethe *et al* (20) showed that the *Mycobacterium smegmatis* laminin-binding protein (which is also expressed by *M. tuberculosis*) binds to laminin.

Using ELISA and reverse ELISA, Kinhikar *et al* (21) demonstrated that purified recombinant malate synthase bound to fibronectin and laminin. This was confirmed by fractionating the recombinant protein using sodium dodecyl sulphate polyacrylamide gel electrophoresis, treating the Western blots with these ECM proteins, and detecting ECM protein binding using anti-ECM and secondary antibodies. These researchers also showed that recombinant malate synthase adhered to epithelial cells. This was done by detecting bound malate synthase using anti-malate synthase antibodies and flow cytometry. Using a similar immunoblotting strategy to Kinhikar *et al* (21), Espitia *et al* (22) and Xolalpa *et al* (23) found that the fractionated recombinant Rv1759c PE-PGRS protein and glutamine synthetase A1 bound to fibronectin, respectively.

Microsphere adhesion assays

Although microspheres have generally been used in *M. tuberculosis* phagocytosis assays, they also have application in the study of bacterial adhesion. Esparza *et al* (24) used 1 μ m green fluorescent polystyrene microbeads coated with purified PstS-1 ABC phosphate

Table 1. A list of the currently known *Mycobacterium tuberculosis* adhesins

Adhesin	Gene (s)	Mediates adhesion to	Reference (s)
19-kDa antigen	<i>Rv3763</i>	Monocytes and macrophages	(24, 25)
Alanine- and proline-rich antigen (Apa)	<i>Rv1860</i>	Pulmonary surfactant protein A and macrophages	(24, 35)
Antigen 85 complex	<i>Rv0129c, Rv1886c, Rv3803c, and Rv3804c</i>	Fibronectin and macrophages	(17, 24)
Cpn60.2 molecular chaperone	<i>Rv0440</i>	Macrophages	(16, 36)
Curli pili	<i>Rv3312A</i>	Laminin, <i>M. tuberculosis</i> , macrophages, and epithelial cells	(13, 14, 19, 28)
DnaK molecular chaperone	<i>Rv0350</i>	Macrophages	(36)
Early secreted antigen ESAT-6	<i>Rv3875</i>	Laminin	(37)
Glutamine synthetase A1	<i>Rv2220</i>	Fibronectin	(23)
Glyceraldehyde-3-phosphate dehydrogenase	<i>Rv1436</i>	Possibly fibronectin (as occurs in group A streptococci)	(38)
Heparin-binding hemagglutinin adhesin	<i>Rv0475</i>	<i>M. tuberculosis</i> and epithelial cells	(12, 27)
Laminin-binding protein	<i>Rv2986c</i>	Laminin	(20)
L,D-transpeptidase	<i>Rv0309</i>	Fibronectin and laminin	(18)
Malate synthase	<i>Rv1837c</i>	Fibronectin, laminin, and epithelial cells	(21)
Membrane protein	<i>Rv2599</i>	Collagen, fibronectin, and laminin	(18)
<i>Mycobacterium</i> cell entry-1 protein	<i>Rv0169</i>	Epithelial cells	(26)
N-acetylmuramoyl-L-alanine amidase	<i>Rv3717</i>	Fibronectin and laminin	(18)
PE-PGRS proteins	<i>Rv1759c</i>	Fibronectin	(22)
	<i>Rv1818c</i>	<i>M. tuberculosis</i> and macrophages	(11)
Protein kinase D	<i>Rv0931c</i>	Brain endothelia and laminin	(15)
PstS-1 (38-kDa antigen)	<i>Rv0934</i>	Macrophages	(24)
Type IV pili	<i>Rv3654c-Rv3660c</i>	Possibly macrophages and epithelial cells	(39)

transporter protein to interact with macrophages adhering to glass coverslips. Non-adherent microbeads were removed by washing and adherent microbeads were identified by analyzing randomly selected cells by confocal microscopy. Similarly, Diaz-Silvestre *et al* (25) used this technique to confirm the role of the 19-kDa antigen (LpqH glycolipoprotein) as an adhesin.

Chitale *et al* (26) used 0.3 and 1.1 μm latex beads coated with recombinant *Mycobacterium* cell entry-1 (Mce1) protein to infect HeLa cells cultured on glass

coverslips. Bead association was assessed by light and transmission electron microscopy. The use of 1 μm fluorescent latex beads has also been described, where fluorescence emission gives an indication of bead association (26).

M. tuberculosis auto-aggregation assays

Apart from their function in bacteria-eukaryotic cell interactions, adhesins also mediate cell-to-cell contact between bacterial cells and are, thus, key

Table 2. A list of the putative *Mycobacterium tuberculosis* adhesins identified by computational analyses ^a

Annotation	Putative adhesin-encoding genes
ESAT-6-like proteins	<i>Rv1037c</i> and <i>Rv3890c</i>
Hypothetical proteins	<i>Rv0538, Rv0584, Rv0679c, Rv0988, Rv1115, Rv1116A, Rv1269c, Rv2190c, Rv2730, Rv3036c, Rv3067, Rv3207c, Rv3337, Rv3491, Rv3705c, Rv3811, and Rv3822</i>
Immunogenic protein Mpt64	<i>Rv1980c</i>
Low-molecular-weight antigen CFP2	<i>Rv2376c</i>
Major secreted immunogenic protein Mpt70	<i>Rv2875</i>
Mce-family protein	<i>Rv0590</i>
PE family proteins	<i>Rv1089</i> and <i>Rv1788</i>
PE-PGRS family proteins	<i>Rv0578c, Rv0872c, Rv0977, Rv0980c, Rv1450c, Rv1803c, Rv2126c, Rv2487c, Rv2853, Rv3345c, Rv3507, and Rv3514</i>
Periplasmic phosphate-binding lipoprotein	<i>Rv0932c</i>
Possible chitinase	<i>Rv1987</i>
Possible conserved lipoproteins	<i>Rv1881c</i> and <i>Rv3576</i>
PPE family proteins	<i>Rv0304c, Rv0354c, Rv0755c, Rv1548c, Rv2353c, Rv2356c, and Rv3159c</i>
Probable cutinase precursor	<i>Rv1984c</i>
Probable lipoprotein aminopeptidase	<i>Rv0418</i>
Probable penicillin-binding protein	<i>Rv0050</i>
Soluble secreted antigen Mpt53 precursor	<i>Rv2878c</i>
Superoxide dismutase	<i>Rv3846</i>

^a Putative adhesins identified from Kumar *et al* (18) and the MycobacRV web server (<http://mycobacteriarv.igib.res.in>)

contributors to bacterial aggregation and biofilm formation. Menozzi *et al* (27) reported that incorporating purified HBHA to *M. tuberculosis* cultures resulted in an aggregation of the bacilli, in a dose-dependent manner. Ramsugit *et al* (28) showed that a MTP-deficient *M. tuberculosis* strain displayed a significant reduction in bacterial aggregation and pellicle formation in detergent-free Sauton's media compared with the wild-type strain. Both HBHA and MTP were subsequently shown to also function as adhesins in the host-pathogen interaction (12-14). The identification of adhesins that promote *M. tuberculosis* auto-aggregation could, therefore, enable the identification of potential adhesins that are associated with host interaction.

In silico analyses

Bioinformatics analyses have led to the identification of numerous potential *M. tuberculosis* adhesins. Kumar *et al* (18) screened all of the *M. tuberculosis* H37Rv proteins through several bioinformatics tools and identified 20 novel putative adhesins (Table 2). This was achieved using SPAAN adhesin predictor (29) at a threshold of $P_{ad} > 0.65$, subcellular localization prediction algorithms [LOCtree (30), PSORTb (31), and SubLoc (32)], and removing potential human homologues that were identified by BLAST. This was followed by an argumentation-based approach to process 'claims' and 'attacks' from different algorithms. In addition, further filtering criteria for globular and low-molecular-weight proteins that were not previously reported in the literature were applied. Three of the prospective adhesins identified in that study (Rv0309, Rv2599, and Rv3717) were subsequently experimentally confirmed to bind to ECM proteins (18). This validates the usefulness of computational methods to identify *M. tuberculosis* adhesins.

The MycobacRV Database of Mycobacterial Vaccine Candidates provides information regarding possible adhesins of 22 human pathogenic mycobacterial species and their potential as vaccine candidates (33). This database uses SPAAN (29) at $P_{ad} > 0.6$ and PSORTb (34) at a very stringent cut-off to identify adhesin and adhesin-like proteins. Using the MycobacRV web server (<http://mycobacteriarv.igib.res.in>), 37 novel prospective adhesins were identified in *M. tuberculosis* H37Rv (Table 2).

Conclusion

TB remains a major challenge. Despite global efforts to improve our understanding of *M. tuberculosis* pathogenesis, insufficient knowledge has been gained on the *M. tuberculosis* adherence mechanisms, the first (and arguably the most critical) step in the pathogenesis of this bacterium. Several *M. tuberculosis* adhesins have been identified; however, computational analyses have

predicted a large number of additional adhesin-encoding genes in this pathogen's genome. Several of these genes encode proteins belonging to the PE-PGRS and Pro-Pro-Glu (PPE) protein families. It, therefore, seems likely that key information regarding this organism's adherence mechanisms could be obtained by the study of the adhesin capability of proteins belonging to these families.

With several methods available for the study of *M. tuberculosis* adhesion, as highlighted in this review, identifying novel *M. tuberculosis* adhesins is possible and should be explored further. Advances in gene disruption technologies open up the possibility of screening mutant libraries to identify *M. tuberculosis* genes involved in the pathogen's adherence. Adapting the adhesion methodologies to enable high-throughput screening of libraries of potential anti-adhesion therapeutics is needed and may have value in the control of this ravaging disease.

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