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The *Mycobacterium avium* ESX-5 PPE protein, PPE25-MAV, Interacts with an ESAT-6 Family Protein, MAV_2921, and Localizes to the Bacterial Surface

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Abstract

Previous research has demonstrated that inactivation of the *Mycobacterium avium* gene, PPE25-MAV (MAV-2928), leads to a significant attenuation of virulence in both *in vitro* and *in vivo* models. PPE25-MAV encodes for a PPE family protein, a family from which many members have been implicated in both bacterial virulence and host immune recognition. Recent research has shown that many PPE family proteins are exported by a specialized Type VII secretion system in mycobacteria. In this context, the mechanisms of PPE25-MAV in *M. avium* pathogenesis were investigated. A mycobacterial 2-hybrid system was used to perform a directed search for *M. avium* proteins that interact directly with PPE25-MAV. An interaction was observed between PPE25-MAV and the ESAT-6 family protein, MAV_2921, and was further defined by 2-hybrid analysis of truncated PPE25-MAV, and confirmed by co-immunoprecipitation. Localization of the PPE25-MAV protein was analyzed in *Mycobacterium smegmatis* expressing the recombinant protein and a significant percentage of PPE25-MAV was shown to be exposed at the bacterial surface by surface biotinylation and trypsin protection assays. Finally, transcriptional analysis of PPE25-MAV and its associated operon suggested that nutrient limitation, a condition which occurs in the phagosome, plays a role in regulating expression of the PPE25-MAV gene.

Keywords

Mycobacterium avium; ESX-5; PPE25-MAV; interaction; pathogenesis

INTRODUCTION

A widespread environmental pathogen, *Mycobacterium avium* is the causative agent of avian tuberculosis (33). It is also a major source of disseminated mycobacteriosis in immuno-compromised individuals, such as those with HIV (8). A robust and hardy member

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of the mycobacteria family, it is capable of establishing persistent environmental and biological biofilms and has been isolated from hospital water supplies and even residential showerheads (5, 10). Like the majority of the pathogenic mycobacteria, it is an intracellular parasite, capable of survival and replication inside of the host cell (15). *M. avium* actively subverts the normal host defense response and inhibits phagosome acidification and maturation in macrophages (24). Furthermore, the bacterium modulates host apoptosis and necrosis processes to facilitate macrophage escape and dissemination (7). Such thorough manipulation of the host likely requires a complex system of sensors, structural elements and effectors. Recent research has indicated that one potential candidate, a unique Type VII secretion system (T7SS), likely plays a central role in mycobacterial pathogenesis (1, 36)

Mycobacteria are encapsulated by a thick, covalently cross-linked, lipid-rich cellular envelope (21). Export of proteins through this hydrophobic barrier is facilitated in part by a mycobacterial T7SS, which is encoded as a gene cluster (17). Originally identified in *Mycobacterium tuberculosis* as a region (ESX-1) whose absence contributes to the attenuation of the tuberculosis vaccine strain BCG, genome sequencing has revealed the presence of up to 5 different loci, ESX-1 thru 5, across the *Mycobacterium* genus (17, 23). *M. avium* contains 4 of these loci, ESX-2 thru 5, but contains a deletion covering the entire region encoding ESX-1. ESX-1 has been shown to be essential for exporting virulence factors CFP-10 and ESAT-6, as well as numerous other proteins (19, 25). The ESX-5 locus has also been implicated in pathogenesis. Disruption of the ESX-5 locus inhibits the ability of the bacteria to modulate the macrophage response, but does not appear to affect the ability of the bacteria to escape into the cytosol from the phagosome (2, 34). Recent research has demonstrated that a functional ESX-5 region facilitates the export of a number of PE and PPE family proteins (3, 4).

PE and PPE proteins, defined by their conserved N-terminal PE (Pro-Glu) and PPE (Pro-Pro-Glu) domains, are a mycobacterium specific family of proteins. PE and PPE family proteins all contain a well conserved N-terminal region (90 and 180 residues, respectively) but have considerable variation among their C-terminal domains (18). They are highly expanded in pathogenic mycobacteria, in some cases comprising almost 10% of the genome (13). Several PE and PPE family proteins are secreted during culture in vitro and during intracellular growth in macrophages (2, 4). Several members of these families associate with or are found within the mycobacterial cell envelope (32). It has been speculated that their abundance, C-terminal variations and apparent redundancy play a role in immune evasion (26). Indeed a number of PE and PPE proteins have been shown to be robust antigenic targets, and in some cases, effective immunizing agents (29, 30).

The objective of this study was to characterize the function of the *M. avium* PPE family gene, MAV_2928. A homologue of the *M. tuberculosis* gene Rv1787, it is situated within the ESX-5 region of *M. avium* (18). Previous research has shown that disruption of this gene abrogates the ability of the bacterium to prevent phagosome acidification and survive within the macrophage (24). Here we utilize a mycobacterial 2-hybrid system (M-PFC) and protein co-precipitation to show that MAV_2928 interacts with the adjacent ESAT family gene, MAV_2921, via its C-terminal domain. In addition, we utilized a cell surface biotinylation approach to show that MAV_2928 localizes to the bacterial surface, in a translocation dependent on its conserved N-terminal domain. Finally, Real-Time PCR analysis of MAV_2928 under various conditions indicates that gene expression is contingent upon nutrient poor conditions. In total, this data supports a model whereby *Mycobacterium avium* compensates for its lack of ESX-1 dependent export by utilizing its PPE/PE export apparatus to secrete CFP/ESAT family proteins.

MATERIALS AND METHODS

2-Hybrid (M-PFC) Vectors

Vectors PUAB100, PUAB200, PUAB300 (prey vector) and PUAB400 (bait vector) were a generous gift from the Steyn lab (Suppl. Table 1). The M-PFC system is a 2-hybrid system optimized for use in the model mycobacterial organism, *Mycobacterium smegmatis* (35). Positive interactions are assessed based on resistance to trimethoprim, which is conferred by interaction between two murine Dihydrofolate Reductase (DHFR) subunits, encoded by the bait (PUAB400) and prey vectors (PUAB300), respectively. To screen for potential interactions between the MAV_2928 protein and other members of the ESX-5 region of *M. avium*, a bait plasmid was created by cloning the 3 gene operon containing MAV_2928, MAV_2927 and MAV_2926 into PUAB400 (Suppl. Table 1). The C-terminus of the DHFR sub-unit was translationally fused to the N-terminus of MAV_2928. To create a directed prey library for a directed search, individual genes from the *M. avium* ESX-5 region were cloned into the PUAB300 vector. This library contained full length genes MAV_2915 through MAV_2933 (Suppl. Table 1). To further define the identified interactions, the MAV_2928 gene and four truncations of MAV_2928 (MAV_2928Δ(186-421), MAV_2928Δ(1-179, 326-421), MAV_2928Δ(1-219) and MAV_2928Δ(1-399)) were cloned into the PUAB300 vector and screened against the prey library (Suppl. Table 1). Empty vectors PUAB300 and PUAB400 were used as negative controls, while MAV_2922 and MAV_2921 were cloned into both the PUAB300 and PUAB400 vectors for use as positive controls.

2-Hybrid Bait Culture Preparation

PUAB400 bait vectors were cultured and purified from *Escherichia coli*. Vectors were transformed into *M. smegmatis* MC²155 via electroporation, as previously described (14). Briefly, actively growing cultures of *M. smegmatis* were inoculated into 100 ml of Middlebrook 7H9 broth supplemented with 10% OADC (Oleic Acid, Albumin, Dextrose and Catalase), (Hardy Diagnostics, Santa Maria, CA). Broth cultures were harvested at mid-log phase (OD₆₀₀~.6) by centrifugation at 4°C. Samples were washed twice in electroporation buffer (10% Glycerol, .05% Tween-20, .05% tween-80). Samples were then re-suspended in 5 ml electroporation buffer. Two hundred µl aliquots were combined with 5 µl plasmid (500 ng/µl) and electroporated. After electroporation, samples were rescued for 2.5 h in 1 ml LB broth media. Rescued samples were then plated on Middlebrook 7H10 agar supplemented with 10% OADC and kanamycin (40 mg/ml) and cultured at 37°C for 5 days. The resulting colonies were screened for the appropriate insert by colony PCR and confirmed by direct sequencing (CGRB Core Facility, Oregon State University).

2-Hybrid Interaction Screen

M. smegmatis cultures containing the appropriate PUAB400 bait construct were cultured in Middlebrook 7H9 broth supplemented with 10% OADC and 40 µg/ml kanamycin and prepared for electroporation as described above. Individual bait plasmids were cultured in *E. coli* and plasmids were purified according to manufacturer's protocol (Qiagen, Alameda, CA). Purified plasmids, covering MAV_2915 thru MAV_2933 in PUAB300, were pooled with a final concentration of 500 ng/µl. Cultures were electroporated and recovered as previously described. Transformed *M. smegmatis* were plated on Middlebrook 7H10 agar plated supplemented with 10% OADC, 40 µg/ml of kanamycin, 50 µg/ml of hygromycin and 20 µg/ml of trimethoprim. Cultures were grown for 4–7 days at 37°C. Resistant colonies were re-streaked onto fresh triple-selective plates. Actively growing cultures were analyzed via colony PCR, plasmids and inserts were confirmed via direct sequencing.

6X-His Tagged Protein Co-Precipitation

To confirm the results observed in the M-PFC screen, the direct interaction of MAV_2928 with MAV_2921 was examined by protein co-precipitation. Full length MAV_2928 (1-421) and three truncations of MAV_2928 (MAV_2928Δ(186-421), MAV_2928Δ(1-179, 326-421) and MAV_2928Δ(1-219)) were cloned into the vector PMV261-5HRFP (Suppl. Fig. 1B, Suppl. Table 1). Full length MAV_2928 and its truncations were cloned as C-terminal fusions to a monomeric RFP moiety with an N-terminus 6X-His tag. The prey vector, PMV261-MAV_2921, was constructed by cloning an unlabeled MAV_2921 into the PMV261 vector. Vector construction, screening and confirmation were performed in *E. coli*. Completed vectors were confirmed via colony PCR and direct sequencing. Vectors were transformed into *M. smegmatis*, as described above. Transformed *M. smegmatis* was cultured on Middlebrook 7H10 agar plates (supplemented with 10% OADC and 50 µg/ml kanamycin) for 5 days at 37°C, then cultured an additional two days at 25°C to allow accumulation of recombinant protein. Expression of recombinant protein was confirmed visually by fluorescent microscopy and by anti-6X-His Western blot (Santa Cruz Biotechnology, Santa Cruz, CA). Samples of 150 mg of bacteria expressing the bait vectors were harvested, washed once with Hank's Buffered Salt Solution (HBSS, Invitrogen, Carlsbad, CA) and resuspended in 1.5 ml denaturing buffer (500 mM NaCl, 7M urea, 20 mM Tris-HCl, 10 mM imidazole, .5% (v/v) glycerol, .2% (v/v) TritonX-100, 1 mM PMSF, pH 8). Bacteria were mechanically disrupted in a bead mill with .1 mm silica beads (6 cycles, 20 s each @ max speed). Samples were centrifuged at 12,000 × g for 5 min and supernatant was removed to a clean tube. Fifty µl of Ni²⁺ coated magnetic beads (Promega, Madison, WI) were added to each sample. Samples were incubated on a rotating mixer at 4°C for 2 h. After incubation, samples were washed three times in denaturing buffer. Each sample was split into two aliquots, one for exposure to the prey lysate and the other for exposure to the control lysate, *M. smegmatis* with an unmodified PMV261 plasmid (Suppl. Table 1). Lysate from *M. smegmatis* carrying the PMV261-MAV_2921 prey construct (containing unlabeled MAV_2921), was prepared in a urea-based denaturing buffer, as described above. One and a half ml of prey lysate was added to one aliquot of each sample containing immobilized bait protein. Proteins were allowed to refold together through sequential dilution of the denaturing buffer with HBSS. Samples were diluted by addition of 1 ml aliquots of HBSS (pH 7.4, 10 mM imidazole), followed by 5 min incubations, until the concentration of urea reached 350 mM (sample volume 30 ml). Proteins immobilized on magnetic beads were purified with a magnetic rack (Promega) and washed three times with HBSS. The beads were resuspended in Laemmli buffer (+5% BME) and proteins were separated by SDS-PAGE. Proteins were stained with Coomassie blue stain and visualized. The band corresponding to the putative MAV_2921 protein was isolated and processed by in-gel trypsin digest (Pierce, Rockford, IL) prior to mass spectrometry analysis.

Mass Spectrometric Sequencing

Trypsin digested protein samples were processed at the Environmental Health Science Center's (EHSC) Mass Spectrometry Facility at Oregon State University (Corvallis, OR). Proteins were analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Tandem MS was performed with electrospray ionization (ESI) and mass spectra were acquired by using quadrupole-time of flight (Q-TOF) Global Ultima system from Micromass (Manchester, UK), operated with a spray voltage of 3.5 kV. Digested protein samples were mixed 1:1 (v/v) with .1% formic acid, .005% trifluoroacetic acid, and 3% acetonitrile in H₂O (Solvent A). A symmetry 300 C-18 trap (Dionex, Sunnyvale, CA) and 75 mm PicoFrit column (New Objective, Woburn, MA), packed with Jupiter C-5 (Phenomenex, Torrance, CA), were used for the ESI experiments. The LC program consisted of a gradient from 3% to 35% Solvent B (.1% formic acid, .005% trifluoroacetic acid in 80% acetonitrile), to 70% at 65 min, and finally to 95% Solvent B at 80 min. Data-

dependent MS/MS was generated using a .5 second MS survey scan and 2.5 second MS/MS scans on the three most abundant peaks found in the survey scan. A database search was performed using Mascot software (Matrix Science, London, UK).

Bacterial Cell Fractionation

To characterize the sub-cellular localization of MAV_2928, full length MAV_2928 (1-421) and three truncations (MAV_2928 Δ (201-421), MAV_2928 Δ (1-179, 326-421) and MAV_2928 Δ (1-259)) were cloned into the PMV261-6X-His vector (Suppl. Fig. 1A, Suppl. Table 1). Vectors were electroporated into *M. smegmatis* and cultured according to the above protocol. To extract soluble proteins, *M. smegmatis* cultures were harvested, washed once with HBSS, re-suspended in HBSS and mechanically disrupted, as previously described. Soluble protein was removed by three extractions in HBSS. The pellet remaining after HBSS extraction was re-suspended in a urea-based denaturing buffer and mechanically disrupted again. Samples were centrifuged at $12,000 \times g$ for 10 min and the supernatant, containing most of the insoluble protein, was harvested. Protein samples were resolved via SDS-PAGE. Proteins were transferred to nitrocellulose membranes and were analyzed via anti-6X-His Western blot, following manufacturer protocols (Santa Cruz Biotechnology, Santa Cruz, CA).

Bacterial Cell Surface Biotinylation

To further define the localization of MAV_2928 and its associated truncations, bacteria were biotinylated to identify surface exposed proteins. *M. smegmatis* cultures carrying the appropriate vectors were treated with sulfo-NHS-LC-biotin (Pierce, Rockford, IL), a membrane impermeable biotinylation reagent targeting primary amines, according to manufacturer protocols. Briefly, 100 mg of bacteria were washed twice in HBSS and resuspended in 1 ml of HBSS and sulfo-NHS-LC-Biotin at a concentration of 2 mM. Cells were incubated for 20 min with shaking at RT then washed three times in a quenching buffer (HBSS + 50 mM glycine). Bacteria were mechanically disrupted in denaturing buffer and proteins were separated via SDS-PAGE, as described above. Proteins were transferred to nitrocellulose and analyzed via anti-6X-His and anti-Biotin Western blot (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were visualized on an Odyssey scanner (Licor, Lincoln, NE). An IRDye800 anti-rabbit IgG secondary antibody (Licor) was used to visualize the anti-6X-His antibody, while an IRDye680-Streptavidin conjugate (Licor) was used to visualize biotinylation.

Trypsin Protection Assay

To confirm the surface localization of MAV_2928, a sample of *M. smegmatis* expressing the PMV261-6XHis-MAV_2928/2927/2926 vector was subjected to limited trypsin proteolysis to digest surface exposed proteins (Suppl. Table 3). For this assay, an N-terminal 6X-His-tagged mRFP molecule, which remains soluble in the cytoplasm, was used as a control (Suppl. Fig. 1). To prepare the sample for trypsin treatment, one turbid ($OD_{600} = 3$) 1 ml sample of *M. smegmatis* carrying PMV261-6XHis-MAV_2928/2927/2926 was mixed with 1 ml of turbid control bacterium (*M. smegmatis* carrying PMV261-6XHis-mRFP) to create 2 ml mixtures. Two 500 μ l aliquots were transferred to clean 1.7 ml tubes from each mixture. One hundred and twenty-five μ g of sequencing grade trypsin was added to one aliquot from each group and allowed to incubate for 30 min at 37°C. The samples were then spun down, washed twice with HBSS and resuspended in denaturing buffer and mechanically disrupted in a bead mill with .1 mM silica beads. Proteins were separated via SDS PAGE and transferred to a nitrocellulose membrane analyzed via Western blot with an anti-6XHis antibody (Santa Cruz Biotechnology). Samples were imaged and analyzed with an

IRDye800 anti-Rabbit secondary antibody and Odyssey scanner, (Licor) and photon emission averages were recorded for each band to quantify the signal intensity.

Fluorescent Microscopy

Cultures of *M. smegmatis* expressing mRFP fusions of MAV_2928, MAV_2922/2921 and mRFP alone (Suppl. Table 1) were visualized on a Leica DM4000B fluorescent microscope (Leica, Wetzlar, Germany).

Mycobacterial RNA Extraction

To isolate RNA from *M. avium*, 100 mg samples of bacteria were harvested and washed once with cold HBSS. Bacteria was mechanically disrupted with a bead beater and .1 mm silica beads in an acidic detergent solution (125 mM NaAcetate, 1% Triton X-100, .6% SDS pH 4.2) and processed through three cycles of phase separation with acid phenol (Acid Phenol: Chloroform 5:1). Samples were then phase separated against Chloroform:Isoamyl alcohol 24:1. RNA was precipitated with ethanol and resuspended in H₂O. RNA concentration was analyzed via Nanodrop (Nanodrop, Wilmington, DE) and adjusted to a final concentration of 200 ng/μl. These samples were then subjected to DNase I treatment (10 U/100 μl DNase I + 1.5 μl RNase inhibitor) at 37°C for 30 min (Invitrogen). After DNase I treatment, RNA was re-isolated using Trizol, following manufacturer protocol (Invitrogen). Final RNA samples were quantified by nanodrop and screened for DNA contamination by PCR.

Real-Time PCR

One hundred ml of mid-log phase culture of *M. avium* was harvested from Middlebrook 7H9 media and washed twice with cold wash buffer (10% glycerol, .1% tween-20). Samples were resuspended in water and aliquoted into flasks containing 100 ml of either 7H9 + 10% OADC (pH 6.8), 7H9 + 10% OADC (pH 5.8), 7H9 + 10% OADC (pH 7.8), 7H9 + 10% OADC + 1 mM Fe²⁺, Saline (diH₂O + 150 mM NaCl) or diH₂O. Samples were cultured in the respective media for 12 h at 37°C with shaking. Samples were harvested by refrigerated centrifugation and processed as described above. One μg of total bacteria RNA was used to generate cDNA using cDNA Superscript 3 Supermix (Invitrogen, Carlsbad, CA), according to manufacturer protocol. Real-Time PCR was performed on an iCycle RT-PCR machine (BioRad, Hercules, CA), using Sybr Green RT-PCR mastermix, according to manufacturer protocol (BioRad). Real-Time PCR results were normalized to an average of two endogenous controls, 16s rRNA and RNA Polymerase Subunit A (RpoA). Results were generated from three biological replicates, each with two experimental replicates. To determine efficiency co-efficient of each reaction and primer set, control samples using *M. avium* genomic DNA at .01 ng/μl, 1 ng/μl and 10 ng/μl were analyzed. For Real-Time PCR primers, see Suppl. Table 4.

MAV_2928 Operon Analysis

The presence of MAV_2928 and MAV_2926 on the same transcript was confirmed by inter-gene PCR from cDNA generated for RT-PCR analysis. A reverse transcriptase enzyme negative cDNA prep was used as a negative control. The primers used were the forward primer for MAV_2928 and reverse primer for MAV_2926 used for RT-PCR analysis (Suppl. Table 4). Bioinformatics analysis of potential operons and terminator sites was performed using *Softberry* software (Softberry, Mount Kisco, NY).

Statistical Analysis

Each experiment was repeated three times and two technical replicates were performed for each of the three experimental replicates. Technical replicates were averaged to yield a

single value for each experimental replicate. The results were expressed as the mean $\pm$ SD. Variance between experimental groups was assessed by one-way ANOVA. A P-value of < 0.05 was considered significant.

RESULTS

MAV_2928 interacts directly with MAV_2921 via its C-terminal domain

To identify proteins that directly interact with MAV_2928, a bait vector (PUAB400) containing the MAV_2928 gene was screened against a pool of potential interaction partners, proteins from the ESX-5 region of *M. avium*, using a mycobacterium based 2-hybrid system (M-PFC) (35). In the initial screen, the downstream genes MAV_2927 and MAV_2926, which form a putative operon with MAV_2928, were included in the bait vector insert. The prey vectors screened included all of the conserved proteins comprising the ESX-5 region (MAV_2933-MAV_2915) of *M. avium* (Suppl. Table 1). While the initial screen generated several positive colonies, sequencing of those colonies revealed that they originated from only one positive interaction, between PPE25-MAV and MAV_2921, which encodes an ESAT-6 like protein. Subsequent screening of the single gene, PPE25-MAV (1-421), and truncations of PPE25-MAV (PPE25-MAV Δ (186-421), PPE25-MAV Δ (1-179, 326-421), PPE25-MAV Δ (1-219) and PPE25-MAV Δ (1-399)), indicated that the interaction between PPE25-MAV and MAV_2921 is mediated by the C-terminal 200 amino acids of PPE25-MAV. The interaction between the C-terminal of PPE25-MAV (residues 220-421) and MAV_2921 resulted in robust growth, as measured by growth rate, while the interaction between full length PPE25-MAV (1-421) and MAV_2921 resulted in notably slower growth. A secondary screen of the pool of prey vectors using a bait protein containing MAV_2921, revealed positive interactions with both PPE25-MAV and MAV_2922. This interaction between MAV_2922 and MAV_2921, which are CFP-10/ESAT-6 family genes, and the standard control vectors, PUAB100 and PUAB200, were used as positive controls. The results for the interaction M-PFC 2-hybrid screen between PPE25-MAV and MAV_2921 are summarized in Table 1 and Figure 1A.

Protein Co-Precipitation

To verify the interactions observed in the 2-hybrid screen, recombinant proteins comprising full length PPE25-MAV (residues 1-421), PPE25-MAV Δ (186-421), PPE25-MAV Δ (1-179, 326-421) and PPE25-MAV Δ (1-219) were generated in *M. smegmatis*. To facilitate ease of expression and purification, as well as to replicate the spatial orientation of proteins from the 2-hybrid screen, all of the bait constructs were generated as C-terminal fusions to a 6X-His tagged monomeric RFP moiety (Suppl. Table 1) and were expressed in *M. smegmatis*. Because of difficulties solubilizing and purifying some constructs in native conditions, recombinant proteins were extracted in denaturing conditions and captured on paramagnetic nickel beads. After thorough washing, the beads were re-suspended in denaturing buffer with *M. smegmatis* lysate containing over-expressed, unlabeled MAV_2921 expressed in the PMV261 vector. *M. smegmatis* lysate from cultures carrying the unmodified expression vector (PMV261) were used as the negative control. Bait proteins were allowed to refold in the presence of excess MAV_2921 through sequential dilution of the denaturant. After thorough washing in HBSS, protein laden beads were re-suspended in laemmli buffer, boiled and separated via SDS-PAGE. Protein bands that appeared consistent with MAV_2921 were excised and positively identified as MAV_2921 by mass spectrometry (Suppl. Table 6). The results suggest direct interactions of PPE25-MAV and PPE25-MAV Δ (1-219) with MAV_2921, but no apparent interaction between MAV_2921 and PPE25-MAV Δ (186-421), PPE25-MAV Δ (1-179, 326-421) or 6X-HIS-mRFP (neg control) (Fig. 1B).

PPE25-MAV is Found Primarily in the Insoluble Bacterial Fraction

Several studies have indicated that many PPE family proteins are either exported from the bacterium or localized to the cell envelope. A recent analysis of *Mycobacterium paratuberculosis*, revealed that a well conserved ESX-5 homologue of PPE25-MAV, MAP1506, was exposed at the bacterial surface (27). To assess whether this is also true for PPE25-MAV, N-terminal 6X-His tagged constructs of full length and truncated PPE25-MAV were generated in *M. smegmatis*. PPE25-MAV was expressed as an operon (PPE25-MAV/2927/2926), a single gene, PPE25-MAV, and several truncations PPE25-MAV Δ (201-421), PPE25-MAV Δ (1-179, 326-421) and (PPE25-MAV Δ (1-259) (Suppl. Table 1). Bacteria expressing recombinant protein were mechanically disrupted and all HBSS soluble protein was extracted. Protein remaining in the insoluble fraction was extracted in a denaturing buffer containing urea. Protein fractions were separated via SDS-PAGE and analyzed by Western blot to determine the solubility of the various protein constructs. The results indicate that the full length PPE25-MAV is almost entirely in the insoluble fraction and both the N-terminal (residues 1-200) and C-terminal (residues 260-421) were seen predominantly in the insoluble fraction. However, the middle truncation (residues 180-320) and the mRFP control localize primarily to the soluble fraction (Fig. 2).

Trypsin Shaving Suggests MAV_2928 is Surface Exposed

To investigate whether MAV_2928 is exposed at the bacterial surface, *M. smegmatis* expressing MAV_2928/2927/2926 with a 6X-His tag at the N-terminus (Suppl. Table 1), was subjected to limited trypsin proteolysis to remove surface exposed proteins. After digest, recombinant *M. smegmatis* was mechanically disrupted in denaturing buffer and the proteins were separated on SDS-PAGE. Semi-quantitative Western blotting demonstrated a significant decrease in labeled MAV_2928 after trypsin digest when compared to a cytoplasmic control (mRFP) (Fig. 3B). This result indicates that a significant percentage of the MAV_2928 protein expressed was located at the bacterial surface, with at least the N-terminal 6X-His tag exposed to the extra-cellular environment.

Cell Surface Biotinylation Indicates MAV_2928 is Surface Exposed in an N terminal Dependent Manner

To further analyze the localization of PPE25-MAV, 6X-His tagged constructs expressed in *M. smegmatis* cultures were treated with Sulfo-NHS-LC-Biotin to label surface exposed proteins. Bacteria were then washed and the proteins isolated by mechanical disruption in a urea-based denaturing buffer. Recombinant proteins were purified from each sample using paramagnetic nickel beads. Whole cell lysate samples and affinity-purified recombinant proteins were then separated on SDS-PAGE gel and analyzed by Western blot for the presence of 6X-His (green) and biotin (red) (Fig. 3A). The results indicate that both the complete PPE25-MAV protein and PPE25-MAV Δ (201-421) are exposed on the cell surface, while PPE25-MAV Δ (1-179, 326-421), PPE25-MAV Δ (1-259) and mRFP (control) are not expressed on the bacterial surface. Consistent with several previous observations regarding the trafficking of PPE family proteins (9, 20, 27, 31, 32), this result suggests that MAV_2928 is translocated to the bacterial surface, likely in a N-terminus dependent process (4).

MAV_2928 Localizes to the Polar Region

Previous research has demonstrated that members of the ESX clusters, including PPE family proteins have a tendency to localize to the polar region of the bacterium (9). To assess whether MAV_2928 localizes in a similar fashion, *M. smegmatis* cultures of three 6XHis-mRFP-fusion constructs (mRFP only, mRFP-MAV_2928 and mRFP-MAV_2922/2921) were visualized via fluorescent microscopy (Fig. 3C). A significant proportion of the mRFP-

MAV_2928 expressing bacteria exhibited the aforementioned polar localization, although some did not show high degrees of localization. Interestingly, the mRFP-MAV_2922/2921 construct, which encodes the CFP-10/ESAT-6 family proteins of ESX-5, appeared to localize evenly to the cell wall. However, while these proteins are soluble, they did not appear to be secreted or surface exposed (data not shown). These data suggest that the MAV_2922/2921 complex may associate with the cell membrane or cell envelope but is not surface-exposed in our model system. It is unclear whether these proteins are normally retained, or are only fully exported in mycobacteria that contain a functional ESX-5 region. The mRFP control was robustly expressed and remained evenly distributed in the bacterial cytoplasm.

PPE25-MAV is Transcribed in an Operon with MAV_2927 and MAV_2926

While the putative structural components of the ESX-5 region are highly conserved amongst pathogenic mycobacteria, the PPE and PE family proteins within the ESX-5 region are highly variable. PPE25-MAV is immediately adjacent to a truncated PE family gene, MAV_2927, whose full length homologue is present in *M. tuberculosis* (Rv1788) but completely deleted in *M. paratuberculosis* (Fig. 4A, 4B). Interestingly, PPE25-MAV is 56 residues longer than its homologue in *M. tuberculosis*, Rv1787 (421 aa to 365 aa), which is the exact size of the truncation in MAV_2927 compared to its homologue, Rv1788 (43 aa to 99 aa). While at least one PPE protein has been shown to form a heterodimer with an adjacent PE protein (Insert Strong 2006 Citation), the operonic organization of PPE and PE proteins has fueled speculation that many of these proteins form similar heterodimers. This potential transfer of information from MAV_2927 to PPE25-MAV suggests a consolidation of function into a single protein unit and raises the possibility that PPE25-MAV may not require a PE pair for proper folding or function. Complicating this analysis, *M. avium* has a total of three additional PPE family genes in the immediate vicinity to PPE25-MAV, all of which are close homologues. In contrast, *M. tuberculosis* has only 2 additional PPE family proteins, while *M. paratuberculosis* has one additional PPE family protein. Analysis with operon finding software (Softberry, Mount Kisco, NY) suggests that PPE25-MAV, MAV_2927 and MAV_2926 are produced on a single polycistronic transcript. Furthermore, a bioinformatics search for putative terminators revealed the presence of a robust Rho-independent terminator site ~50 base pairs downstream of the stop codon for MAV_2926 (Fig. 4C). Inter-gene PCR between PPE25-MAV and MAV_2926, using *M. avium* cDNA as a template, confirmed that these three genes were present on the same transcript, likely constituting an operon (Fig. 4C, Suppl. Table 4).

The MAV_2928 Operon is Upregulated in Response to Nutrient Limited Conditions

Previous research has indicated that MAV_2928 is minimally expressed in normal in vitro culture conditions, but is up-regulated after phagocytosis (24). To better understand what factors might be involved in regulating this response, expression of PPE25-MAV and MAV_2926 were analyzed by Real-Time PCR following several culture conditions. Levels of transcript of these two genes were assessed in *M. avium* 109 after 12 h of culture in standard media (7H9 + 10% OADC, pH 6.8), standard media supplemented with 1 mM Fe²⁺, acidified standard media (pH 5.8), basified standard media (pH 7.8), saline (diH₂O + 150 mM NaCl) and diH₂O. Real-Time results indicated that all culture conditions utilizing standard media produced minimal expression levels of PPE25-MAV and MAV_2926, consistent with previous research (24). However, both the saline and diH₂O exhibited a moderate, albeit significant increase in expression of the PPE25-MAV operon (Fig. 5). This result suggests that expression of PPE25-MAV and MAV_2926 is repressed in the presence of nutrients, ions, lipids or other factors which are less abundant in the early phagosome. Interestingly, it was observed in preliminary screens of other PPE (MAV_2925, MAV_2924, MAV_2914 and MAV_2913), PE (MAV_2923 and MAV_2915) and CFP/

ESAT (MAV_2922 and MAV_2921) family genes associated with the ESX-5 region, that while the CFP/ESAT family genes were robustly expressed in all conditions, the PPE and PE family genes appeared to be differentially regulated (data not shown). This result suggests that *M. avium* may modulate the ratios between closely related ESX-5-associated PPE and PE family proteins in response to changing environmental conditions.

DISCUSSION

Pathogenic mycobacteria are known to subvert their hosts by exporting proteins that interfere with host immune recognition and response systems (1, 2). To facilitate export of these proteins through thick, lipid-rich cell envelopes, the genomes of pathogenic mycobacteria species encode up to five copies (ESX1-5) of a specialized Type Seven Secretion System (T7SS). Mycobacterial T7SS systems have been shown to be necessary for export of several families of proteins, including PPE, PE, ESAT-6/CFP-10 homologues (4, 12, 19). In many cases, disruptions in either the T7SS systems or their substrates have been shown to diminish bacterial fitness, decrease virulence or cause lethality (2, 18, 34). In this study, we investigated the mechanisms by which a PPE gene, MAV_2928, promotes intracellular survival of *M. avium*. Expanding on previous data showing that a loss of MAV_2928 inhibits the intracellular viability of *M. avium* (22, 24), our data indicate that PPE25-MAV directly interacts with an ESAT-6 family protein, MAV_2921, that PPE25-MAV is translocated to the bacterial surface and that it is expressed in response to the nutrient limited conditions found in the phagosome. This data indicates that PPE25-MAV may play a role in mediating interaction with the host at the bacteria-phagosome interface, either directly, through its association with MAV_2921 or through additional mechanisms.

Pathogenic mycobacteria contain up to five well-conserved loci encoding T7SSs, ESX1-5. Of these five, both ESX-1 and ESX-5 have been shown to play important roles in bacterial pathogenesis (3, 17, 19). ESX-1, the most extensively studied loci, appears responsible for secretion of small soluble proteins, such as the CFP-10/ESAT-6 dimer and EspA (12, 16), while ESX-5 has been shown to be responsible for export of several PPE and PE family proteins (2, 4), many of which are quite large and poorly soluble. Interestingly, *M. avium* lacks the ESX-1 region, but is able to maintain virulence, in contrast to *Mycobacterium marinum* and *M. tuberculosis* strains that have undergone a disruption in their respective ESX-1 regions (23). Two-hybrid screening of potential interactions between PPE25-MAV and other members of the ESX-5 loci revealed an interaction with MAV_2921, but failed to identify additional proteins as binding partners. The interaction between the *M. tuberculosis* PPE protein, Rv3873, and the ESAT-6 protein, Rv3875, which are both components of the ESX-1 region, has been previously described (28, 38). While Rv3873 and Rv3875 differ from PPE25-MAV and MAV_2921, this new data supports the possibility of a functional relationship between PPE and ESAT family proteins within their respective ESX loci. Furthermore, 2-hybrid and in-vitro co-precipitation analysis of PPE25-MAV truncations suggest that this protein-protein interaction is mediated by the variable C-terminal domain of PPE25-MAV.

While the highly varied nature of the C-terminus of PPE and PE family proteins suggest a diverse array of functions, both PPE and PE proteins are characterized by a highly conserved N-terminal domain (18). X-ray crystallography of the *M. tuberculosis* PE/PPE pair, Rv2431c/30c, revealed that both the PPE and PE motifs are likely exposed (37). Previous research has indicated that these motifs may represent signal sequences or be chaperone binding sites involved in protein export (6, 11). This conclusion is supported by the work of Abdallah and colleagues, who demonstrated the necessity of a functional ESX-5 region in the export of several members of the PPE and PE families (2–4). However, since all mycobacteria encode PPE and PE proteins, yet not all mycobacteria have both ESX-1 and

ESX-5 regions, it appears possible that there is some diversity and overlap of export substrates between the various ESX loci. Saprophytic mycobacteria generally lack the ESX-5 region and encode fewer PPE and PE family genes, and those retained genes tend to be ancestral homologues located within the ESX loci (18). *M. smegmatis* lacks the ESX-5 loci and has been shown to be deficient in the export of Rv2430c/2431c, a well-defined soluble PPE/PE pair that is absent in *M. avium* (3). In contrast to that observation, the protease protection and surface biotinylation experiments performed in this study indicate that PPE25-MAV is exported to the surface of *M. smegmatis* where it associates with the mycobacterial cellular envelope. The possibility that the surface localization of PPE25-MAV in *M. smegmatis* results from incomplete secretion due to the lack of a functional ESX-5 region, remains to be explored. Conversely, while these PPE family proteins vary in sequence, solubility and structure, this result may also support an alternative processing and export pathway, perhaps utilizing the apparatus encoded by the ancestral ESX-1 loci. Consistent with previous observations, analysis of PPE25-MAV truncations demonstrates that the translocation of PPE25-MAV is dependent on the highly conserved N-terminal domain of the protein. Cell fractionation analysis shows that PPE25-MAV is present predominantly in the insoluble fraction, likely firmly complexed with components of the cell envelope.

Previous studies have demonstrated that co-expression of many PPE proteins and their cognate PE proteins leads to the production of a soluble heterodimer (37). However, in our 2-hybrid analysis, PPE25-MAV failed to show a positive interaction with either of the two PE proteins in close proximity, MAV_2927 and MAV_2923 (data not shown). Furthermore, expression of the PPE25-MAV/2927/2926 operon (Fig. 2), as well as co-expression of PPE25-MAV with both of the proximate PE proteins, MAV_2927 and MAV_2923, did not lead to a soluble product in either *M. smegmatis* or *E. coli* (data not shown). This result is consistent with bioinformatics analysis that indicates that the majority of expected surface exposed residues of PPE25-MAV, based on structural homology with other PPE family proteins, are non polar amino acids (data not shown). In total, our data suggests that PPE25-MAV is present at the bacterial surface, likely embedded in the bacterial envelope.

Bioinformatics analysis of PPE25-MAV suggested that PPE25-MAV is transcribed on a polycistronic transcript that also contains a PE family protein, MAV_2927 and a PPE family protein, MAV2926. This possibility was confirmed by inter-gene PCR demonstrating a transcriptional linkage. This operon is followed by an energetically robust transcriptional terminator loop, making further downstream transcription unlikely. Comparison between *M. tuberculosis*, *M. avium* and *M. paratuberculosis* revealed a rapidly evolving region within the highly stable ESX-5 loci. Comparison between *M. avium*, *M. paratuberculosis* and *M. tuberculosis*, suggest a potential transfer of function from MAV_2927 to PPE25-MAV. The MAV_2927 protein (43 aa) is 56 residues smaller than its *M. tuberculosis* homolog, Rv1788 (99 aa), which is exactly how much larger PPE25-MAV (421 aa) is in relation to its homolog, Rv1787 (365 aa). Furthermore, the elimination of the MAV_2927 homolog in *M. paratuberculosis*, which also has the larger species of the PPE25-MAV homolog, MAP1505 (421 aa), suggests that MAV_2927 may be unnecessary or redundant. The deletion in MAV_2927 is expected to remove the predicted alpha-helical structure that has been shown to be important for complexing a PE protein with its coordinate PPE protein (37). Taken together, these observations raise the possibility that the PPE protein PPE25-MAV may not require a cognate PE protein to assume its mature conformation or function.

Upregulation of MAV_2928 at the transcriptional level has been previously observed during intra-cellular growth of *M. avium* (24). To better understand the conditions within the phagosome that trigger upregulation of PPE25-MAV, expression of PPE25-MAV and the co-expressed gene MAV_2926 were assessed via Real-Time PCR in response to pH

changes, iron supplementation and nutrient deprivation. Interestingly, a condition well known to drive changes in gene expression, phagosomal acidification, appeared to have minimal effect on levels of PPE25-MAV and MAV_2926 transcription. Instead, PPE25-MAV and MAV_2926 were significantly upregulated in response to complete nutrient deprivation, both in a hypotonic and isotonic environment. It was also observed in preliminary screens that while the CFP-10 and ESAT-6 family proteins, MAV_2922 and MAV_2921, were robustly expressed, the other PPE and PE family genes in the ESX-5 region were differentially expressed in response to the conditions assayed. While this data requires additional validation, it is consistent with a model in which environmental changes modulate the ratios of closely related PPE and PE family proteins. This modulation may play a role in evasion of the host system, be involved in adaptation to new environments, nutrient acquisition or have a completely novel function.

In conclusion, the data presented here provides some new insights into previous observations that have shown the necessity of the ESX-5 region in mycobacterial virulence. In *M. avium*, the loss of virulence and intracellular survival associated with an inactivation of PPE25-MAV has provided an important area of focus that could yield new drug targets capable of diminishing *M. avium* pathogenesis. Although *M. avium* differs markedly from other pathogenic mycobacteria, similar results from the disruption of the ESX-5 region in other mycobacterial species suggest a critical role for this well-conserved T7SS loci. Our data contributes to previous observations of interactions between PPE and ESAT family proteins, and expands on the potential role of PPE25-MAV in *M. avium* pathogenesis. The direct interaction between these proteins may be especially important for *M. avium*, as it lacks the ESX-1 loci, which is thought to encode the primary export system for CFP/ESAT family proteins in most pathogenic mycobacteria. With extensive duplications, large overall populations and well conserved protein structures, it appears likely that protein interactions between PPE, PE, CFP and ESAT family proteins are more promiscuous than their operonic organization would suggest. It is possible that this enormous potential complexity underwrites the incredible success of *M. avium* and other pathogenic mycobacteria as pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T7SS	type seven secretion system
M-PFC	mycobacterial protein fragment complementation
DHFR	dihydrofolate reductase
mRFP	monomeric red fluorescent protein

REFERENCE LIST

1. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmek BJ, Bitter W. Type VII secretion--mycobacteria show the way. *Nat Rev Microbiol*. 2007; 5:883–91. [PubMed: 17922044]
2. Abdallah AM, Savage ND, van Zon M, Wilson L, Vandenbroucke-Grauls CM, van der Wel NN, Ottenhoff TH, Bitter W. The ESX-5 secretion system of *Mycobacterium marinum* modulates the macrophage response. *J Immunol*. 2008; 181:7166–75. [PubMed: 18981138]
3. Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, Eisenberg D, Musters RJ, Vandenbroucke-Grauls CM, Appelmek BJ, Luirink J, Bitter W. A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. *Mol Microbiol*. 2006; 62:667–79. [PubMed: 17076665]
4. Abdallah AM, Verboom T, Weerdenburg EM, Gey van Pittius NC, Mahasha PW, Jimenez C, Parra M, Cadieux N, Brennan MJ, Appelmek BJ, Bitter W. PPE and PE_PGRS proteins of *Mycobacterium marinum* are transported via the type VII secretion system ESX-5. *Mol Microbiol*. 2009; 73:329–40. [PubMed: 19602152]
5. Aronson T, Holtzman A, Glover N, Boian M, Froman S, Berlin OG, Hill H, Stelma G Jr. Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. *J Clin Microbiol*. 1999; 37:1008–12. [PubMed: 10074518]
6. Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, Cole ST. Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? *Mol Microbiol*. 2002; 44:9–19. [PubMed: 11967065]
7. Bermudez L, Danelishvili L, Early J. Mycobacteria and macrophage apoptosis: complex struggle for survival. *Microbe*. 2006; 1:372–375.
8. Brodt HR, Enzensberger R, Kamps BS, Keul HG, Helm EB. Impact of disseminated *Mycobacterium avium*-complex infection on survival of HIV-infected patients. *Eur J Med Res*. 1997; 2:106–10. [PubMed: 9113499]
9. Carlsson F, Joshi SA, Rangell L, Brown EJ. Polar localization of virulence-related Esx-1 secretion in mycobacteria. *PLoS Pathog*. 2009; 5:e1000285. [PubMed: 19180234]
10. Carter G, Wu M, Drummond DC, Bermudez LE. Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*. *J Med Microbiol*. 2003; 52:747–52. [PubMed: 12909649]
11. Cascioferro A, Delogu G, Colone M, Sali M, Stringaro A, Arancia G, Fadda G, Palu G, Manganelli R. PE is a functional domain responsible for protein translocation and localization on mycobacterial cell wall. *Mol Microbiol*. 2007; 66:1536–47. [PubMed: 18028308]
12. Champion PA, Stanley SA, Champion MM, Brown EJ, Cox JS. C-terminal signal sequence promotes virulence factor secretion in *Mycobacterium tuberculosis*. *Science*. 2006; 313:1632–6. [PubMed: 16973880]
13. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*. 1998; 393:537–44. [PubMed: 9634230]
14. Danelishvili L, Poort MJ, Bermudez LE. Identification of *Mycobacterium avium* genes up-regulated in cultured macrophages and in mice. *FEMS Microbiol Lett*. 2004; 239:41–9. [PubMed: 15451099]
15. Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. *Proc Natl Acad Sci U S A*. 2007; 104:11038–43. [PubMed: 17578930]
16. Fortune SM, Jaeger A, Sarracino DA, Chase MR, Sasseti CM, Sherman DR, Bloom BR, Rubin EJ. Mutually dependent secretion of proteins required for mycobacterial virulence. *Proc Natl Acad Sci U S A*. 2005; 102:10676–81. [PubMed: 16030141]

17. Gey Van Pittius NC, Gamielidien J, Hide W, Brown GD, Siezen RJ, Beyers AD. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol.* 2001; 2:RESEARCH0044. [PubMed: 11597336]
18. Gey van Pittius NC, Sampson SL, Lee H, Kim Y, van Helden PD, Warren RM. Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. *BMC Evol Biol.* 2006; 6:95. [PubMed: 17105670]
19. Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR. Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol.* 2004; 51:359–70. [PubMed: 14756778]
20. He Z, De Buck J. Localization of proteins in the cell wall of *Mycobacterium avium* subsp. paratuberculosis K10 by proteomic analysis. *Proteome Sci.* 8:21. [PubMed: 20377898]
21. Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H. Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc Natl Acad Sci U S A.* 2008; 105:3963–7. [PubMed: 18316738]
22. Jha SS, Danelishvili L, Wagner D, Maser J, Li YJ, Moric I, Vogt S, Yamazaki Y, Lai B, Bermudez LE. Virulence-related *Mycobacterium avium* subsp *hominissuis* MAV_2928 gene is associated with vacuole remodeling in macrophages. *BMC Microbiol.* 2010; 10:100. [PubMed: 20359357]
23. Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, Sherman DR. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. *J Infect Dis.* 2003; 187:117–23. [PubMed: 12508154]
24. Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE. A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cell Microbiol.* 2005; 7:539–48. [PubMed: 15760454]
25. MacGurn JA, Raghavan S, Stanley SA, Cox JS. A non-RD1 gene cluster is required for Snm secretion in *Mycobacterium tuberculosis*. *Mol Microbiol.* 2005; 57:1653–63. [PubMed: 16135231]
26. McEvoy CR, van Helden PD, Warren RM, Gey van Pittius NC. Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis* PPE38 gene region. *BMC Evol Biol.* 2009; 9:237. [PubMed: 19769792]
27. Newton V, McKenna SL, De Buck J. Presence of PPE proteins in *Mycobacterium avium* subsp. paratuberculosis isolates and their immunogenicity in cattle. *Vet Microbiol.* 2009; 135:394–400. [PubMed: 19004580]
28. Okkels LM, Andersen P. Protein-protein interactions of proteins from the ESAT-6 family of *Mycobacterium tuberculosis*. *J Bacteriol.* 2004; 186:2487–91. [PubMed: 15060053]
29. Okkels LM, Brock I, Follmann F, Agger EM, Arend SM, Ottenhoff TH, Oftung F, Rosenkrands I, Andersen P. PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. *Infect Immun.* 2003; 71:6116–23. [PubMed: 14573626]
30. Parra M, Cadieux N, Pickett T, Dheenadhayalan V, Brennan MJ. A PE protein expressed by *Mycobacterium avium* is an effective T-cell immunogen. *Infect Immun.* 2006; 74:786–9. [PubMed: 16369041]
31. Sampson SL, Lukey P, Warren RM, van Helden PD, Richardson M, Everett MJ. Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. *Tuberculosis (Edinb).* 2001; 81:305–17. [PubMed: 11800581]
32. Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jimenez CR, Daffe M, Appelmek BJ, Bitter W, van der Wel N, Peters PJ. Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathog.* 2010; 6:e1000794. [PubMed: 20221442]
33. Schaefer WB, Beer JV, Wood NA, Boughton E, Jenkins PA, Marks J. A bacteriological study of endemic tuberculosis in birds. *J Hyg (Lond).* 1973; 71:549–57. [PubMed: 4518354]
34. Simeone R, Bottai D, Brosch R. ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol.* 2009; 12:4–10. [PubMed: 19155186]

35. Singh A, Mai D, Kumar A, Steyn AJ. Dissecting virulence pathways of *Mycobacterium tuberculosis* through protein-protein association. *Proc Natl Acad Sci U S A*. 2006; 103:11346–51. [PubMed: 16844784]
36. Stanley SA, Raghavan S, Hwang WW, Cox JS. Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *Proc Natl Acad Sci U S A*. 2003; 100:13001–6. [PubMed: 14557536]
37. Strong M, Sawaya MR, Wang S, Phillips M, Cascio D, Eisenberg D. Toward the structural genomics of complexes: crystal structure of a PE/PPE protein complex from *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2006; 103:8060–5. [PubMed: 16690741]
38. Teutschbein J, Schumann G, Mollmann U, Grabley S, Cole ST, Munder T. A protein linkage map of the ESAT-6 secretion system 1 (ESX-1) of *Mycobacterium tuberculosis*. *Microbiol Res*. 2009; 164:253–9. [PubMed: 17433643]

FIG. 1. Interaction between PPE25-MAV and MAV_2921 in mycobacterial 2-hybrid system. **A.** Growth of M-PFC *M. smegmatis* colonies with dual plasmids (PUAB300-Prey and PUAB400-Bait) on triple selective media (kanamycin, hygromycin and trimethoprim). Positive interactions between fusion proteins encoded on each plasmid confer resistance to trimethoprim. **B.** Protein co-precipitation identifying PPE25-MAV C-terminus as responsible for interaction with MAV_2921. Bait proteins were expressed in *M. smegmatis* with N-terminal 6X-Histidine tags purified on magnetic beads under denaturing conditions. Bait proteins are mRFP only (a), mRFP-PPE25-MAV (full length, residues: 1-421) (b), mRFP- PPE25-MAV Δ (186-421) (N terminus, residues: 1-185) (c), mRFP- PPE25-MAV Δ (1-179, 326-421) (Mid protein, residues: 180-325) (d), and mRFP PPE25-MAV Δ (1-219) (C terminus, residues: 220-421) (e). Bait proteins were allowed to refold in the presence of *M. smegmatis* urea-soluble lysate from cultures containing either the empty PMV261 vector (left) or PMV261-MAV_2921 (right), encoding an unlabeled copy of the ESAT-6 like protein, MAV_2921.

FIG. 2.

Cell fractionation shows that PPE25-MAV is primarily found in the insoluble fraction of *M. smegmatis*. Western blot analysis of soluble and insoluble fractions of *M. smegmatis* expressing N-terminal 6X-His tagged proteins. Soluble proteins were first extracted by mechanical disruption in HBSS. Insoluble proteins were then extracted in a denaturing urea solution. Cell envelope and membrane localizing proteins tend to remain insoluble in HBSS, while cytoplasmic and secreted proteins are readily extracted in HBSS. Proteins analyzed: (a) mRFP only, (b) mRFP-PPE25-MAV/MAV_2927/MAV_2926 operon, (c) PPE25-MAV (full length, residues: 1-421), (d) PPE25-MAVΔ(201-421) (N terminus, residues: 1-200), (e) PPE25-MAVΔ(1-179, 326-421) (Mid Protein, residues: 185-325), (f) PPE25-MAVΔ(1-259) (C terminus, residues: 260-421).

A

B

C

FIG. 3.

Localization of PPE25-MAV to the surface of the mycobacterial cell envelope. **A.** Western blot demonstrating limited trypsin proteolysis of bacterial surface proteins cleaves the 6X-His tag on PPE25-MAV. Samples of *M. smegmatis* expressing N-terminal 6X-His tagged PPE25-MAV/2927/2926 (operon) were subjected to brief trypsin proteolysis to digest surface exposed residues. Samples of *M. smegmatis* expressing 6X-His-mRFP only, a cytoplasmic protein, were used as a control for cell lysis. Samples are visualized and quantified via semi-quantitative Western blot on the Licor Odyssey Platform. **B.** Surface biotinylation of *M. smegmatis* expressing PPE25-MAV causes labeling of PPE25-MAV. Samples of *M. smegmatis* expressing N-terminal 6X-His tagged proteins were surface labeled with sulfo-NHS-LC-biotin. Total protein from each sample was separated and analyzed by Western blot for biotinylation (red) and 6X-His moiety (green). Analyzed samples: (a) PPE25-MAV (full length, residues: 1-421), (b) PPE25-MAV Δ (201-421) (N terminus, residues: 1-200), (c) PPE25-MAV Δ (1-179, 326-421) (Mid Protein, residues: 180-325), (d) PPE25-MAV Δ (1-259) (C terminus, residues: 260-421), and (e) WT *M. smegmatis*. **C.** Fluorescent microscopy protein localization of mRFP fusion proteins. Intrabacterial localization of fusions: mRFP only, mRFP-PPE25-MAV (full length, residues: 1-421) and mRFP-MAV_2922/2921. Note the polar localization of PPE25-MAV and inner membrane localization of MAV_2922/2921, in contrast to the cytoplasmic mRFP.

FIG. 4. PPE25-MAV, MAV_2927 and MAV_2926 are organized in a polycistronic operon. **A.** Comparative organization of the PPE25-MAV loci in the ESX-5 region of *M. avium* and the homologous region in *M. tuberculosis* and *M. paratuberculosis*. Diagram indicates the truncation of the *M. avium* PE protein, MAV_2927, compared to its *M. tuberculosis* homologue, Rv1788. The diagram also indicates the position of the Rho-independent terminator (stem loop icon) that concludes the PPE25-MAV operon. **B.** Inter-gene PCR from *M. avium* cDNA indicates PPE25-MAV and PPE25-MAV are on the same transcript. The forward primer is located within the PPE25-MAV gene and the reverse primer is located in the MAV_2926 gene. The negative control is a reverse transcriptase negative sample of the same cDNA preparation. **C.** Visualization and free energy value of the stem loop terminator located immediately downstream of the MAV_2926 gene. This structure terminates the polycistronic transcript that encodes PPE25-MAV, MAV_2927 and MAV_2926.

FIG. 5.

Real-Time PCR analysis of PPE25-MAV and MAV_2926 expression in response to different culture conditions. Real-Time PCR gene expression values for PPE25-MAV and MAV_2927. Samples were cultured for 12 h at 37°C in liquid media. Results represent the averages of three experimental replicates. Data was standardized to two controls, 16S RNA and RNA polymerase A. A P value of < .05 is indicated by *.

TABLE 1

Summary of interactions from the MAV_2928 2-Hybrid Screen against components of the ESX-5 region of *M. avium*.

PUAB400 Bait Vector	PUAB300 Empty	PUAB300-MAV_2921	PUAB300-MAV_2922
PUAB400 Empty	Neg	Neg	Neg
PPE25-MAV/27/26	Neg	Pos	Neg
PPE25-MAV	Neg	Pos	Neg
PPE25-MAVΔ(186-421)	Neg	Neg	Neg
PPE25-MAVΔ(1-179, 326-421)	Neg	Neg	Neg
PPE25-MAVΔ(1-219)	Neg	Pos	Neg
PPE25-MAVΔ(1-399)	Neg	Neg	Neg
MAV_2921	Neg	Neg	Pos
MAV_2922	Neg	Pos	Neg

Neg: No growth

Pos: Growth