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Novel Virulence Factor Secretion Systems in Mycobacteria

by

Scott Edward Converse

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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ACKNOWLEDGMENTS

I would like to begin by thanking Jeff Cox for being an amazing mentor and friend. Jeff's contagious excitement about science was immediately recognizable back in 2000, as he described to me the numerous projects available in his nascent lab. Our common interest in all things Lynchian further aided in my decision to join the first group of students in his lab. I estimate that I have spent over two hundred hours in Jeff's office during the course of the last 5 years, either charting the direction of my project or carefully preparing manuscripts. I have always sought to emulate his meticulous and logical writing style, and our experience writing together has been the single most important instruction I have received at UCSF. Furthermore, our mutual fondness for hours-long brain-storming sessions (with the occasional rampant speculation) made our meetings both enjoyable and incredibly instructive. Jeff has an amazing ability to "connect the dots" and bring together diverse research to the problem of tuberculosis, and his critical thinking skills have been particularly useful in my development as a scientist.

I would also like to thank Peter and Ron for their guidance over the past 4 years. I have been consistently in awe of their research and knowledge, and they have made extremely valuable suggestions about the direction of my project. During my first meeting with Peter, I was struck by something he said, so I immediately returned to lab, wrote it on a Post-It note, and taped it to my computer monitor, where it has remained ever since. Peter said, "Establish

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causality not correlation.” I will never forget these words as I enter into the world of science policy.

I would especially like to thank the inaugural class of the Cox Lab: Jason, Madhulika, Sarah, and Sridharan. You all have been a wonderful support network for me, and your dedication to science (and karaoke) has been truly inspirational. I would also like to thank everyone in the Cox lab over the years that have helped make the lab such a great place to work.

I have also been blessed with fruitful collaborators from our neighbors across the Bay: UC-Berkeley. As the sulfolipid project neared completion, we learned that Carolyn Bertozzi and Julie Leary at UCB were also studying sulfolipid. Rather than “circle the wagons,” we decided to get together, share our findings, and pool our resources. Their two students, Joseph Mougous and Mike Leavell, provided invaluable guidance to my project, and their mass spectrometric skills were instrumental in the publication of my first paper. I would also like to thank Gordon Vrdoljak, Reena Zalpuri, and Kent McDonald at the UCB-Electron Microscopy Lab for their expert instruction and guidance with the EM work of my project. In particular, I would like to thank Gordon for the beautiful pictures of *M. smegmatis* in Chapter 4 and for affording me the opportunity to finally meet my bug face-to-face.

In the early days of the Cox lab, we began meeting with Anita Sil's group who also share our interest in intracellular pathogens. These meetings have sparked numerous new directions and ideas for my project, and I would like to thank Anita in particular for her advice and guidance during my qualifying exam

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A vertical strip of a book cover, likely from a University of California publication. The cover features a repeating pattern of the University of California logo, which consists of a stylized 'U' and 'C' with the words 'UNIVERSITY OF CALIFORNIA' written below it. The pattern is repeated vertically along the entire length of the strip. The background color is a light, neutral tone.

preparation as I prepared to switch to a new project: Snm secretion in *M. smegmatis*.

Last, but most certainly not least, I would like to thank those dearest to me. First, I want to thank Laura for her consistent encouragement and endless patience. Her strong support throughout the past four years has helped me overcome the self-doubt and disappointment that inevitably occur during graduate school. Laura is a perfect example of a hard-working leader, and she has a unique ability to deeply connect with people from all walks of life. I look forward to our coming years together and seeing all of her accomplishments. Finally, I would like to thank my parents, sister, and grandparents; I have blessed with such a loving family, and their emphasis on education and unflagging support for me opened up a world of opportunity, for which I am eternally grateful.

The text of Chapter 2 is a reprint of the material as it appears in "MmpL8 is required for sulfolipid-1 biosynthesis and *Mycobacterium tuberculosis* virulence." Converse, S.E., Mougous, J.D., Leavell, M.D., Leary, J.A., Bertozzi, C.R., and Cox, J.S. Proceedings of the National Academy of Sciences, May 13, 2003, 100(10):6121-6. The text of Chapter 3 is a reprint of the material as it appears in "A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*." Converse, S.E., Cox, J.S. Journal of Bacteriology Feb 2005;187(4):1238-45.

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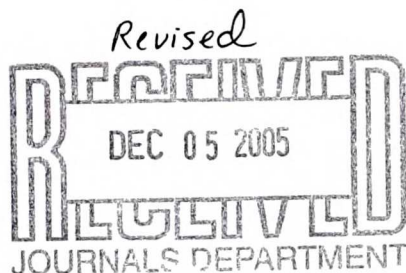


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December 5, 2005

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ABSTRACT

Novel Virulence Factor Secretion Systems in Mycobacteria

Scott E. Converse

Mycobacterium tuberculosis is a remarkably successful human pathogen, and the mechanisms used to manipulate the host cell and evade host defenses are poorly understood. However, there exists clear evidence that the cell wall of *M. tuberculosis* plays a critical role during pathogenesis. Its elaborate structure and diverse components function not only as a defensive barrier, but as a specialized delivery system for virulence factors. My work on mycobacteria details two novel secretion systems responsible for exporting virulence factors outside the bacterial cell. The first pathway, involving the MmpL8 lipid transporter, functions to synthesize and secrete a complex surface-exposed sulfated glycolipid, termed sulfolipid. In the absence of MmpL8, a partially acylated SL-1 precursor, which we term SL₁₂₇₈, accumulates inside the cell. Importantly, MmpL8 is strictly required for virulence in the mouse model of infection, which we attribute to the accumulation of SL₁₂₇₈ *in vivo*. The second pathway, secretion in mycobacteria or Snm, involves an alternative protein secretion pathway, which delivers multiple effectors across the cell wall and into the extracellular milieu. Interestingly, this secretion system is conserved across numerous Gram-positive bacteria, and we show that this pathway is conserved and functional in the non-pathogenic saprophyte *Mycobacterium smegmatis*. Using a genetic and biochemical approach, we

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have uncovered four new components of the Snm secretion pathway which localize to distinct cellular compartments. These studies not only provide important details to our model of the Snm pathway, but develop a useful model system to understand the secretion pathway at a mechanistic level.

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Chapter 1

Introduction

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Mycobacterium tuberculosis was first discovered as the etiological agent of human tuberculosis infection in 1882, and it was immediately recognized that its defining characteristic was its elaborate cell wall. Its unique acid-fast staining suggested novel structures, distinct from the typical Gram-positive or Gram-negative classification. Many features of the mycobacterial cell wall were eventually elucidated, and its remarkable complexity shares characteristics with both Gram-positive and Gram-negative cell walls. There exists four distinct layers of the cell wall: beyond the mycobacterial plasma membrane, there exists the ubiquitous peptidoglycan layer followed by a Gram-positive-like arabinogalactan layer. Mycobacteria have evolved the ability to further modify the arabinogalactan layer by attaching long chain (C₆₀-C₉₀) fatty acids, which form the functional equivalent of the Gram-negative outer membrane. This outer membrane leaflet, named the mycolate layer, serves as the platform for the fourth and outermost layer of the cell wall, a diverse set of lipids, lipoglycans, and proteins which are loosely associated with the mycolate layer, but also mediate important cell-cell and cell-host contacts.

The elaborate cell wall of mycobacteria is responsible for their striking robustness, as it serves as an important defensive barrier during infection. The inherent drug resistance of mycobacteria is due to the cell wall's ability to limit diffusion of antibiotics. As further evidence of its critical defensive role, the effective anti-tuberculosis medications largely target enzymes involved in cell wall biogenesis. However, emerging evidence also implicates the

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mycobacterial cell wall as a system to deliver virulence factors into the host cell during pathogenesis.

The mycobacterial cell wall, due to its novel mycolic acid layer, consists largely of lipid molecules (60% of the total mass), and sequencing of the *M. tuberculosis* genome further confirmed the abundant lipidome of this pathogen (Cole et al., 1998; Tekaia et al., 1999). While *E. coli* possesses ~50 enzymes involved in fatty acid metabolism, *M. tuberculosis* possesses over 250 lipogenic and lipolytic enzymes. This finding further emphasizes the importance of lipid biology to the lifestyle of mycobacteria, and recent work has implicated these cell wall lipid species as critical mechanisms of virulence. Accordingly, numerous genetic screens seeking genes important for *M. tuberculosis* pathogenesis revealed a critical *in vivo* role for cell wall-associated lipid molecules (Camacho et al., 1999; Cox et al., 1999). As these components are loosely associated with mycobacterial cell surface, they likely mediate important host-pathogen interactions during infection.

While the mycobacterial cell wall is an effective defensive barrier, it also presents a unique dilemma to the mycobacterial cell. How do mycobacteria deliver lipid or protein molecules across the four layers of the cell wall? My work in mycobacteria focuses on two novel mechanisms by which mycobacteria have solved the problem of export across its cell wall. The first pathway details the role of MmpL8 in delivering the complex glycolipid, sulfolipid, to its final destination in the outermost layer of the cell wall. Early work to understand the precise component responsible for surface neutral red staining, another unique

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staining feature of mycobacteria, uncovered a novel sulfated lipid species, named sulfolipid. This unique glycolipid, consisting of a sulfated trehalose head group attached to four acyl chains, is an abundant constituent of whole bacteria (0.1%-0.2% of dry bacterial weight) and, more interestingly, strictly found in virulent strains of mycobacteria (Goren, 1970; Goren et al., 1971; Goren et al., 1974a). While the precise role of sulfolipid *in vivo* remains controversial, a rich literature links sulfolipid to various virulence properties of mycobacteria, such as its ability to block host cell phagosome maturation and attenuate host immune signaling events (Goren et al., 1976; Pabst et al., 1988; Zhang et al., 1988; Zhang et al., 1991; Brozna et al., 1991). Our genetic approach allowed us to precisely test the *in vivo* role of sulfolipid and also uncover a surprising role for the MmpL8 lipid transporter in sulfolipid maturation.

My second project details another novel mechanism by which mycobacteria deliver virulence factors, in this case protein effectors, across the cell wall and ultimately into the extracellular milieu where they mediate important host-pathogen interactions. This pathway, which we have named the *Snm* (secretion in mycobacteria) pathway, utilizes a dedicated alternative secretion machine to export two small proteins, named ESAT-6 and CFP-10. These highly immunogenic proteins were long known to be abundant components of the secreted protein profile, despite their lack of apparent signal sequences (Skjot et al., 2000; Sonnenberg and Belisle, 1997; Sorensen et al., 1995). This paradox was solved when several labs uncovered that the genes that surround the *esat6/cfp10* (*esxA/esxB*) operon encode a secretion

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apparatus that delivers ESAT-6 and CFP-10 through the plasma membrane and beyond the cell wall (Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003). This pathway is critically important for mediating host-pathogen interactions *in vivo*, as mutants that cannot synthesis or secrete ESAT-6 and CFP-10 are avirulent in animal models and unable to alter the host environment for their own survival.

Bioinformatic analyses of sequenced microbial genomes revealed that the *snm/esx* genes are widely-conserved in Gram-positive bacteria, including the non-pathogenic relative of *M. tuberculosis*, *Mycobacterium smegmatis* (Gey Van Pittius et al., 2001; Pallen, 2002). In order to understand the mechanism of *Snm* secretion, we took a genetic approach to identify more components of the *Snm* secretion machine in *M. smegmatis*. This strategy has uncovered four more *Snm* secretory proteins which mediate the difficult task of translocating ESAT-6 and CFP-10 through the extensive mycobacterial cell wall. In addition, we have discovered dramatic extracellular filaments and outer membrane vesicles at the surface of the mycobacterial cell wall.

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Chapter 2

MmpL8 is Required for Sulfolipid-1 Biosynthesis and *Mycobacterium tuberculosis* Virulence

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Abstract

Mycobacterium tuberculosis, the causative agent of human tuberculosis, is unique among bacterial pathogens in that it displays a wide array of complex lipids and lipoglycans on its cell surface. One of the more remarkable lipids is a sulfated glycolipid, termed sulfolipid-1 (SL-1), which is thought to mediate specific host-pathogen interactions during infection. However, a direct role for SL-1 in *M. tuberculosis* virulence has not been established. Here we show that MmpL8, a member of a large family of predicted lipid transporters in *M. tuberculosis*, is required for SL-1 production. The accumulation of an SL-1 precursor, termed SL₁₂₇₈, in *mmpL8* mutant cells indicates that MmpL8 is necessary for an intermediate step in the SL-1 biosynthesis pathway. Using a novel fractionation procedure, we demonstrate that SL-1 is present on the cell surface whereas SL₁₂₇₈ is found exclusively in more internal layers. Importantly, we show that *mmpL8* mutants are attenuated for growth in a mouse model of tuberculosis. However, SL-1 *per se* is not required for establishing infection as *pks2* mutants, which are defective in an earlier step in SL-1 biosynthesis, have no obvious growth defect. Thus, we hypothesize that either MmpL8 transports molecules in addition to SL-1 that mediate host-pathogen interactions or the accumulation of SL₁₂₇₈ in *mmpL8* mutant cells interferes with other pathways required for growth during the early stages of infection.

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Introduction

Mycobacterium tuberculosis is an intracellular pathogen that is highly adapted to infect and persist within mammalian tissues (McKinney et al., 1998; Russell, 2001). Although little is known about the molecular mechanisms of *M. tuberculosis* virulence, an emerging paradigm is that cell wall lipids play important roles in the pathogenesis of this organism (Glickman and Jacobs, 2001). The cell wall of *M. tuberculosis* is a complex structure that is responsible for many of the unique properties of the organism (Brennan and Nikaido, 1995). One of the distinguishing features of the mycobacterial cell wall is the extremely long chain fatty acids, termed mycolic acids, which are esterified to the arabinogalactan core. In addition, a highly diverse set of surface-exposed lipids and glycolipids are non-covalently associated with the mycolic acids, creating an asymmetric bilayer structure (Nikaido et al., 1993). Together, these features of the cell wall create a protective barrier that is vital for the survival of the organism within the host.

A rich literature exists that also implicates mycobacterial surface-exposed lipids in modulating specific host-pathogen interactions. Early work established a correlation between levels of two major cell wall lipids, sulfolipid-1 (SL-1, the most abundant sulfatide) and phthiocerol dimycocerosate (PDIM), and the virulence of *M. tuberculosis* (Goren et al., 1974a; Goren et al., 1974b). Indeed, purified SL-1, as well as other mycobacterial lipids such as trehalose 6,6' dimycolate and lipoarabinomannan, have interesting immunomodulatory activity when administered to animals or cultured immune cells (Brozna et al.,

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MmpL7 is a member of a family of 13 predicted lipid transporters encoded by the *M. tuberculosis* genome. Many of these *mmpL* genes are adjacent to polyketide synthase genes in the chromosome, prompting the speculation that individual MmpL proteins transport the lipids produced by the neighboring synthases (Tekaia et al., 1999). Indeed, this hypothesis is supported by the fact that *mmpL7*, *ppsA-E*, and *fadD28* are clustered together

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in the genome. In this study, we describe the identification of another *mmpL* family member, *mmpL8*, and show that this gene, like the neighboring polyketide synthase *pks2* (Sirakova et al., 2001), is essential for SL-1 biosynthesis. Furthermore, we show that although SL-1 is dispensable during the acute stage of infection, *mmpL8* is absolutely required for normal *M. tuberculosis* growth *in vivo*.

Results

Isolation of *mmpL8* transposon mutants from a screen for attenuated strains. In an extension of a previous screen to identify genes involved in virulence of *M. tuberculosis*, we identified two mutants with transposon insertions in the same gene, *mmpL8* (Figure 1A). Because each transposon contained a unique sequence tag, the representation of these mutants within pools of 48 random mutants could be quantified by amplification of all the tags, followed by labeling and hybridization to filter arrays spotted with each individual tag. As shown in Figure 1B, both of these mutants were equally represented in inoculum pools, yet markedly underrepresented after 3 weeks of growth in the lungs of infected mice. These results suggested that *mmpL8* is required for growth during the early stages of infection (see below).

Inspection of the genomic location of *mmpL8* revealed that it is near *pks2*, which encodes a polyketide synthase required for synthesizing phthioceranic and hydroxyphthioceranic acids, the methyl-branched acyl

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constituents of sulfolipid-1 (SL-1) (Sirakova et al., 2001). To study *mmpL8* and *pks2*, we removed the entire open reading frames from a wild-type *M. tuberculosis* strain and confirmed these deletions by Southern blot (Figure 1C). Importantly, deletion of either gene did not affect growth in culture as the doubling time of $\Delta mmpL8$, $\Delta pks2$, and wild-type cells were 20.2 ± 0.3 , 20.2 ± 0.2 , and 20.2 ± 0.3 hours, respectively.

Initial characterization of *mmpL8* role in mycobacterial lipid biosynthesis.

To test the hypothesis that MmpL8 functions in the SL-1 biogenesis pathway, we monitored SL-1 synthesis by labeling cells with ^{14}C -propionate and separating total lipids by thin-layer chromatography (TLC) (Figure 2A). Since both PDIM and SL-1 contain methyl-branched fatty acids, these lipids (as well as other methyl-branched lipids in *M. tuberculosis*) are labeled by ^{14}C -propionate, yet they are distinguishable by their mobility on TLC plates. As expected, SL-1, a major species in wild-type cells, is completely absent in extracts prepared from the $\Delta pks2$ mutants (Figure 2A, lane 2). Reintroduction of the *pks2* gene restored the ability of the strain to synthesize SL-1 (lane 3). Interestingly, we noted a minor species (Lipid 2) that was present in the complemented $\Delta pks2$ strain as well as the wild-type, but was absent in $\Delta pks2$ cells.

Like $\Delta pks2$ mutants, each of the *mmpL8* transposon mutants and the $\Delta mmpL8$ strain failed to synthesize SL-1 (Figure 2A, lanes 4-6). This surprising result was confirmed by mass spectrometry (MS) which demonstrated that SL-1

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was absent in extracts from both $\Delta mmpL8$ and $\Delta pks2$ mutant cells (data not shown). In contrast to the $\Delta pks2$ mutant, however, *mmpL8* mutants accumulated a lipid species that co-migrated with the Lipid 2 species detected in wild-type cells. This defect was due specifically to disruption of *mmpL8* as a single copy of the intact gene fully restored SL-1 synthesis (lane 7). Notably, PDIM synthesis was not perturbed in either *mmpL8* or *pks2* mutants, suggesting that these genes function specifically in SL-1 and Lipid 2 biosynthesis.

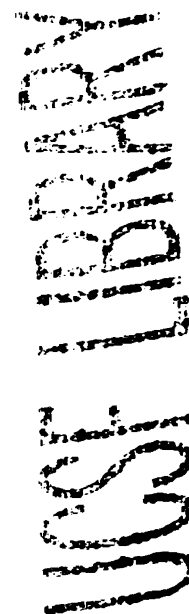
Next, we sought to determine if Lipid 2 and SL-1 share common structural features (see Figure 2C). The observation that Lipid 2 is labeled with ^{14}C -propionate suggests that, like SL-1, this lipid also contains methyl-branched fatty acids (Figure 2A). To determine if Lipid 2 contains a sulfur atom, we labeled both wild-type and $\Delta mmpL8$ cells with $^{35}\text{S-SO}_4^{2-}$ and separated total lipids by TLC. As shown in Figure 2B (lane 1), SL-1 from wild-type cells is labeled strongly with ^{35}S -sulfur, and over-exposure of the TLC revealed that Lipid 2 also contains ^{35}S -sulfur (data not shown). Importantly, the Lipid 2 that accumulates in $\Delta mmpL8$ cells also contains ^{35}S -sulfur (lane 2). As expected, $\Delta pks2$ cells lack both SL-1 and Lipid 2 (lane 3). Taken together, these results confirm that Lipid 2 shares significant structural features with SL-1.

Mass spectrometric analysis of a novel lipid in $\Delta mmpL8$ mutant cells.

To further characterize the structure of Lipid 2, we sought to determine its mass using FT-ICR MS. Spectra from partially purified Lipid 2 were complex, and

included numerous lipid-like molecules (data not shown). Lipids can be identified based on a characteristic 14 Da spacing, which reflects heterogeneity in the acyl chain lengths that vary by $(\text{CH}_2)_n$ units. To distinguish the peaks corresponding to Lipid 2 from contaminants, we utilized a novel approach that combines ^{34}S heavy isotopic labeling with high resolution MS, to identify sulfur-containing compounds from complex mixtures (Mougous et al., 2002b). In short, sulfur-containing molecules isolated from cells grown in the presence of $^{34}\text{SO}_4^{2-}$ will shift by 2 Da as compared to those grown in the presence of $^{32}\text{SO}_4^{2-}$. Using this method on partially purified Lipid 2 from *mmpL8* mutant cells, a group of lipofoms within the m/z range of 1270-1450 shifted by 2 Da in the ^{34}S -labeled sample indicating the presence of a sulfur atom in the underlying structure (Figure 3A & B).

In an effort to assign an elemental composition to Lipid 2, we performed exact mass measurements on the smallest lipofom (m/z 1278, SL_{1278}). The measured mass of SL_{1278} was determined to be m/z 1277.91 and is within 11 ppm of the exact mass of a partially acylated sulfated trehalose (Figure 3C). This structure is consistent with the previously elucidated structure of fully-acylated sulfolipid-1 (Figure 2C). The exact position of the hydroxyphthioceranic acid could not be determined by this approach, but it is likely to be present on either the 6- or 6'-position of trehalose. SL_{1278} was recently identified as a minor sulfated species found in *M. tuberculosis* (Mougous et al., 2002b), consistent with our finding that SL_{1278} is present in minor quantities in wild-type cells (Figure 2A, lane 1).

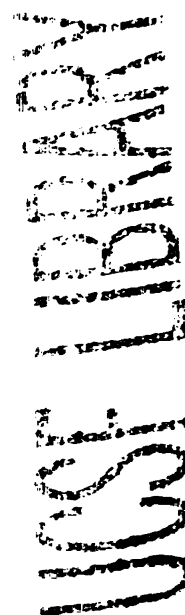


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Biochemical analysis of cell wall lipids in *M. tuberculosis mmpL* mutants.

To determine if SL₁₂₇₈ is a true biosynthetic precursor of SL-1, we performed pulse-chase experiments. Wild-type and *mmpL8* mutant cells were labeled briefly with ¹⁴C-propionate, and total lipids were extracted at various timepoints and analyzed by TLC (Figure 4A). At early timepoints, both wild-type and Δ *mmpL8* mutant cells were capable of synthesizing significant amounts of SL₁₂₇₈. In wild-type cells, radiolabel appeared in SL-1 at later timepoints, while in *mmpL8* mutant cells, radiolabeled SL₁₂₇₈ persisted throughout the timecourse. Taken together these results strongly suggest that SL₁₂₇₈ is a naturally occurring SL-1 precursor and that MmpL8 is required for SL-1 maturation.

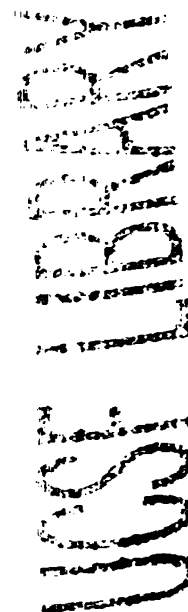
Since MmpL8 is a member of a family of lipid transporters thought to export lipids from the cytoplasmic membrane to the cell wall of *M. tuberculosis*, we sought to determine the subcellular localization of SL-1 and SL₁₂₇₈. To this end, we developed a gentle fractionation procedure in which surface-exposed lipids were extracted from the outer leaflet of the cell wall while lipids that reside in deeper layers remained cell associated. In brief, radioactively labeled cells were suspended in a mixture of mild organic solvents, gently sonicated, and centrifuged. Comparison of solvent-exposed lipids (supernatant, S) to the total lipid sample (pellet, P) allowed us to identify lipids present on the surface of the cell wall.





Previous work has demonstrated that PDIM is normally located on the cell surface in wild-type cells, but present in the cytoplasmic membrane of *mmpL7* mutant cells (Camacho et al., 2001; Cox et al., 1999). Therefore, to validate our new procedure, we determined the subcellular localization of PDIM in both wild-type and *mmpL7* mutant cells (Figure 4B). As expected, large amounts of PDIM was detected in the supernatant fraction from wild-type cells (lanes 1-2, bottom panel). The presence of PDIM in the pellet fraction is likely a consequence of the gentle extraction procedure, resulting in incomplete extraction of surface-exposed lipids. Importantly, PDIM was found exclusively in the cell pellet fraction of *mmpL7* mutant cells, in agreement with MmpL7's proposed function as a PDIM transporter (lanes 3-4, bottom panel). This result demonstrates the utility of this assay for probing the exterior portions of the cell wall for the presence of lipids.

Next, we sought to determine the subcellular localization of SL-1 and SL₁₂₇₈ in wild-type and Δ *mmpL8* mutant cells. Based upon our pulse-chase experiments, we labeled cells for 2 h in order to visualize both species by TLC. In wild-type cells, SL-1 is found in the supernatant fraction, demonstrating that this lipid is present on the cell surface, whereas SL₁₂₇₈ is never detected on the cell surface (Figure 4B, lanes 1-2). Likewise, in Δ *mmpL8* cells, SL₁₂₇₈ is found exclusively in the cell pellet fraction, suggesting that it resides within interior portions of the cell (Figure 4B, lanes 5-6). SL-1 secretion is fully restored in the *mmpL8* complemented strain (Figure 4B, lanes 7-8). To ensure that SL₁₂₇₈ is soluble in the extraction solvent, we performed harsher, lytic extraction

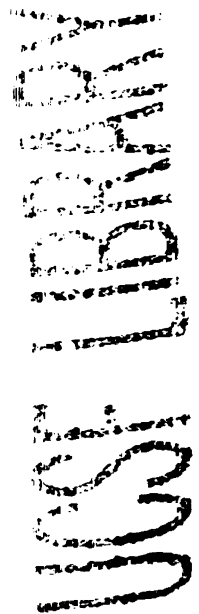


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procedures and found abundant levels of SL₁₂₇₈ in the supernatant fractions from wild-type and $\Delta mmpL8$ mutant cells (data not shown). Importantly, PDIM secretion is normal in $\Delta mmpL8$ mutant cells while SL-1 synthesis and secretion is normal in $\Delta mmpL7$ mutant cells, demonstrating that these two MmpL family members act on distinct lipid substrates.

Role of MmpL8 and SL-1 in *M. tuberculosis* infection.

The identification of *mmpL8* from our transposon screen suggested that SL-1 is important for *M. tuberculosis* pathogenesis. To test this notion, we infected mice by the intravenous route with wild-type, $\Delta pks2$, and $\Delta mmpL8$ mutant strains and monitored their growth *in vivo*. Surprisingly, $\Delta pks2$ mutant cells grew at rates indistinguishable from that of wild-type cells in the spleen, liver, and lung, indicating that SL-1 is not necessary for *M. tuberculosis* growth during the innate phase of immunity (Figure 5A-C). In marked contrast, $\Delta mmpL8$ mutant cells were attenuated in all of these organs. In the spleen and liver, $\Delta mmpL8$ mutant cells grew initially but, unlike wild-type cells, were rapidly eliminated after the 7-day timepoint. $\Delta mmpL8$ mutants also showed a subtle yet statistically significant growth defect in the lung that resulted in threefold fewer bacteria by 6 weeks after infection compared to wild-type cells (Figure 5C). Taken together, these results demonstrate that, although SL-1 is dispensable for *M. tuberculosis* replication during infection, MmpL8 is required for sustained bacterial growth and persistence.



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Discussion

In this study, we identified *mmpL8* in an unbiased screen for genes required for *M. tuberculosis* virulence. Using this same methodology, we had previously identified a homologous lipid transporter, *mmpL7*, which is required for PDIM secretion and *M. tuberculosis* virulence (Cox et al., 1999). Surprisingly, *mmpL8* mutants show a dramatic SL-1 synthesis defect, whereas *mmpL7* mutants can fully synthesize PDIM and only fail to export it to the cell wall. Furthermore, we have shown that *mmpL8* functions in a step downstream of *pks2* in the SL-1 biosynthetic pathway, as a partially acylated precursor, termed SL₁₂₇₈, accumulated intracellularly in *mmpL8* mutants. Using a powerful new technique to identify sulfated compounds, we were able to assign a molecular structure to this intermediate that is consistent with previous structural analysis of SL-1 (Goren, 1970; Goren et al., 1971). Finally, pulse-chase analysis revealed that this is a natural intermediate in wild-type cells, rather than an aberrant by-product of *mmpL8* mutation.

These results have allowed us to draw a model of SL-1 biosynthesis and the role of *mmpL8* in distal steps of this pathway (Figure 6). Because *pks2* mutants synthesize neither SL-1 nor SL₁₂₇₈, and since SL₁₂₇₈ contains one hydroxyphthioceranic acid, it is likely that Pks2 works in a more proximal step in the pathway. Additional enzymes must also function upstream of MmpL8, as the trehalose head-group of SL₁₂₇₈ has undergone sulfation and two acyltransferase reactions. The enzymes that catalyze these reactions and the order in which they function is unclear. Likely candidates for the sulfation step

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are one or more of the three sulfotransferases encoded by the *M. tuberculosis* genome (Mougous et al., 2002a). It is also tempting to speculate that the other genes in the *mmpL8* genomic region, *papA1* or *fadD23* (Figure 1A), may encode the acyltransferases required for the assembly of SL-1.

Why might MmpL8, a predicted lipid transporter, be required for SL-1 biosynthesis? One possibility is that MmpL8, unlike MmpL7, may have some unknown catalytic activity and directly modifies SL-1 during its production. Perhaps more likely is that MmpL8 plays an indirect role in SL-1 biogenesis, consistent with its proposed lipid transport function. As shown in Figure 6, MmpL8 may flip SL₁₂₇₈ across the plane of the cell membrane, allowing it to be further modified into mature SL-1 before delivery to the cell wall. The enzymes that catalyze these later steps are unknown.

Delivery of lipid intermediates to extracytoplasmic synthases may be a common feature among some members of the MmpL family and homologues in related bacterial species. For example, *Mycobacterium smegmatis* mutants deficient in TmpC, a *mmpL* family member, are unable to synthesize glycopeptidolipid (Recht et al., 2000). Likewise, when the *mmpL* homologue, *actII-ORF3* is disrupted in *Streptomyces coelicolor*, actinorhodin synthesis is altered (Bystrykh et al., 1996). Thus, *mmpL7* mutants may still synthesize mature PDIM because the entire synthesis machinery is localized to the cytoplasmic side of the cell membrane.

In addition to MmpL8's role in SL-1 biosynthesis, we have also shown that MmpL8 is required to establish a high-level infection in mice. We initially

A vertical strip of a book cover, likely a spine or endpaper, featuring a repeating pattern of stylized, calligraphic text in a dark ink on a light background. The text is arranged in a vertical column, with some characters appearing to be part of a larger, larger script. The pattern is consistent and repeats down the length of the strip.

isolated *mmpL8* mutants based on their competitiveness in a pooled infection and subsequently demonstrated that these mutants are defective for growth in all organs tested when infected as a clonal population. Interestingly, *pkc2* mutants, which are also defective for SL-1 biosynthesis, are not attenuated during mouse infection. Thus, despite previous evidence implicating SL-1 in pathogenesis, our results clearly demonstrate that SL-1 *per se* is not required for growth of *M. tuberculosis* during the acute phase of infection in mice. We cannot rule out that SL-1 may be required during chronic infection of mice or perhaps plays a role in human *M. tuberculosis* infection that is not apparent in the mouse model of tuberculosis. However, the clear defect of *mmpL8* mutants suggests that other MmpL8-dependent processes, besides SL-1 synthesis, are important for *M. tuberculosis* to thrive during innate immune responses.

There are several models that could explain the paradoxical *in vivo* growth of *pkc2* and *mmpL8* mutants. The simplest model is that MmpL8 may transport other substrates in addition to SL-1, and it is these molecules that are important for pathogenesis. While we have no direct evidence that argues against this model, using numerous experimental approaches we have not identified additional lipids whose synthesis or secretion is blocked in *mmpL8* mutants. A second model is that the accumulation of SL₁₂₇₈ may be generally toxic to the *mmpL8* mutant cells. However, this effect would have to be manifest only during infection, as *mmpL8* mutants grow with wild-type kinetics in liquid culture. Alternatively, the accumulation of SL₁₂₇₈ in *mmpL8* mutant cells may cause a specific inhibition of other pathways required to avert host

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defenses. For example, the accumulation of the sulfated lipid may inhibit a sulfotransferase, leading to a perturbation in sulfate homeostasis and, as a result, an alteration of host-pathogen interactions. We are currently working to determine if any of these or perhaps other models can explain the phenotypic differences between *mmpL8* and *pks2* mutants.

MmpL8 is the second member of the MmpL lipid transporter family demonstrated to be required for virulence. It is likely that other members of this family of transporters perform critical functions during *M. tuberculosis* virulence. Whether the lipids transported by MmpL proteins play only a structural role in the cell wall or perhaps mediate specific host-pathogen interactions remains to be elucidated. In either case, the MmpL family is an attractive target for the design for new anti-tuberculosis drugs.

Materials and Methods

Media and Strains.

M. tuberculosis cells (Erdman strain) were cultured as described previously (Cox et al., 1999). For MS analysis, cultures were grown in a modified Sauton media (Mougous et al., 2002b). The *mmpL8::Tn5367-1*, *mmpL8::Tn5367-2*, $\Delta mmpL8$, $\Delta pks2$, $\Delta pks2 + ppks2$, and $\Delta mmpL8 + pmmpL8$ strains are named jcm130, jcm131, jcm108, jcm119, scm6, and scm8 respectively.

A vertical strip of a book's endpaper, showing a repeating pattern of a stylized 'U' and 'I' logo, likely a publisher's mark. The pattern is printed in a dark ink on a light-colored paper. The 'U' and 'I' are stylized, with the 'U' having a small 'n' inside it. The 'I' is a simple vertical bar. The pattern repeats down the length of the strip.

STM mutagenesis.

STM mutagenesis, mutant isolation, tag amplification and insertion determination were performed exactly as described previously (Cox et al., 1999).

Construction of *M. tuberculosis* knockout strains and complementation constructs.

The $\Delta mmpL8$ (jcm108) and $\Delta pks2$ (jcm119) mutant strains were created by homologous recombination using specialized transducing phages phjsc311 and phjsc377, respectively (Glickman et al., 2000). These deletions replaced 3254 bp of *mmpL8* (a.a. 5-1089) and 6305 bp of *pks2* (a.a. 14-2116) with a hygromycin resistance cassette. Correct replacement of both genes was confirmed by Southern blot. For $\Delta pks2$, genomic DNA was digested with AatII, and the blot was probed with a 610 bp 5' flank, revealing a 2871 bp fragment for wild-type and a 3918 bp fragment for the mutant. For $\Delta mmpL8$, genomic DNA was digested with SmaI, and the blot was probed with a 709 bp 3' flank, revealing a 2638 bp fragment for wild-type and a 3832 bp fragment for the mutant.

The *pks2* (pSEC7) and *mmpL8* (pSEC16) complementation vectors each contained the entire ORF under the control of the endogenous promoter and were created by subcloning from a cosmid, cERD4F2, into the integrating vector pMV306.kan.

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Biochemical analysis of PDIM and SL-1.

Actively growing cultures were diluted to $OD_{600} = 0.6$ in a final volume of 50mL. 7.5 μ Ci of ^{14}C -propionate was added and incubation continued for 16 h at 37 °C. Cell pellets were extracted essentially as described (Cox et al., 1999). Lipids were separated on 10cm x 10cm HPTLC plates (Alltech) using either hexanes:ether (9:1) to resolve PDIM, or chloroform:methanol:water (60:30:6) to resolve SL-1. Radioactive sulfate experiments were performed as above, except 100 μ Ci of ^{35}S - SO_4^{2-} was incubated with 10mL of cells for 48 hours.

For pulse-chase experiments, the cells were labeled for 30 minutes, pelleted, washed with media, and resuspended in 100 ml of radioactive-free 7H9 media. 10 mL aliquots were removed at different time points and the cells were harvested for lipid extraction.

To analyze surface-exposed lipids from *M. tuberculosis*, 7.5 μ Ci of ^{14}C -propionate was added and incubated for 2 h to strongly label both SL-1 and SL₁₂₇₈. Cell pellets were resuspended in 1 mL hexanes-0.05% decylamine and sonicated using a Branson Sonifier 250 with a two-inch cup horn chamber (Branson Ultrasonics, Danbury, CT) at 25% for 90 seconds. The supernatant and cell pellet were recovered by centrifugation and decylamine removed by extracting 3X with 0.1N HCl. Lipids were extracted and analyzed as described above.

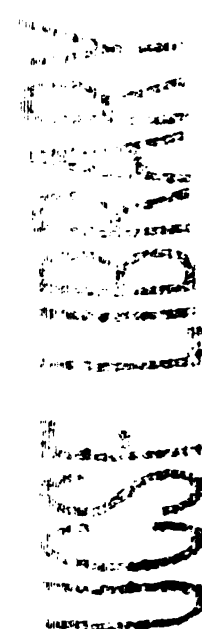
Mass spectrometry.

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Lipid 2 from $\Delta mmpL8$ mutant cells was partially purified using preparative TLC plates and eluted with $\text{CHCl}_3:\text{MeOH}$ (1:2). The recovered lipids were dried under nitrogen and dissolved in $\text{CHCl}_3:\text{MeOH}$ (2:1). Spectra were obtained using a Bruker Apex II FT-ICR MS (Bruker Daltonics, Billerica, MA) equipped with a 7T actively shielded magnet as described previously (Mougous et al., 2002b). Spectra were acquired in the negative ion mode using electrospray ionization, and were composed of 512 k datapoints. Authentic SL-1 was obtained from Colorado State University (NIH, NIAID Contract N01 AI-75320).

Infection of individual *M. tuberculosis* mutants.

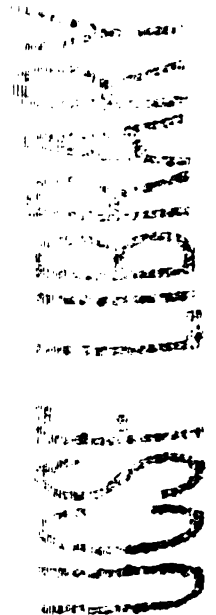
Actively growing wild-type, jcm108, and jcm119 strains were washed, resuspended in 1 x PBS and 0.1% Tween and sonicated. C57BL/6 mice were infected by tail injection with $1-2 \times 10^6$ c.f.u. of each strain using an $\text{OD}_{600} = 3 \times 10^8$ c.f.u. ml^{-1} . Organs from infected mice were homogenized and plated as described (Cox et al., 1999).



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Figure 1. Two *mmpL8* mutants of *M. tuberculosis* identified by STM mutagenesis.

(A) Diagram of *mmpL8* and surrounding genes denoting the transposon insertion sites (1660 and 2238 bp with respect to the start codon) in each mutant. **(B)** Signature DNA tags from mutant mycobacteria harvested from the inoculum and the lungs of 2 mice after 3 weeks of growth were amplified, radiolabelled and hybridized to tag array filters (Cox et al., 1999). Tags from the two underrepresented *mmpL8* mutants are shown next to control tags that did not vary throughout the experiment. **(C)** $\Delta mmpL8$ and $\Delta pks2$ strains were generated by specialized transduction, and Southern blot revealed the expected bands indicating the replacement of the genomic sequence with the hygromycin cassette. For details, see Materials and Methods.



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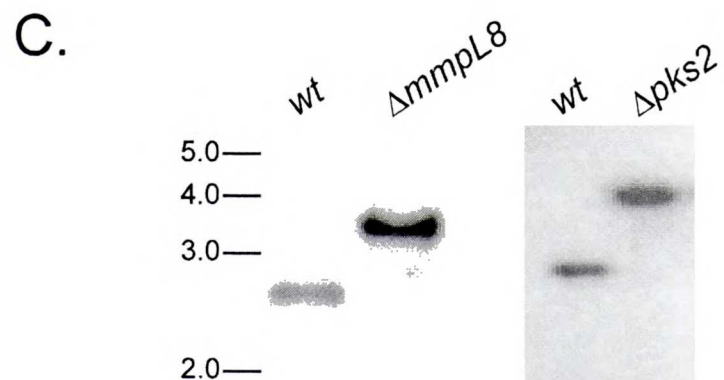
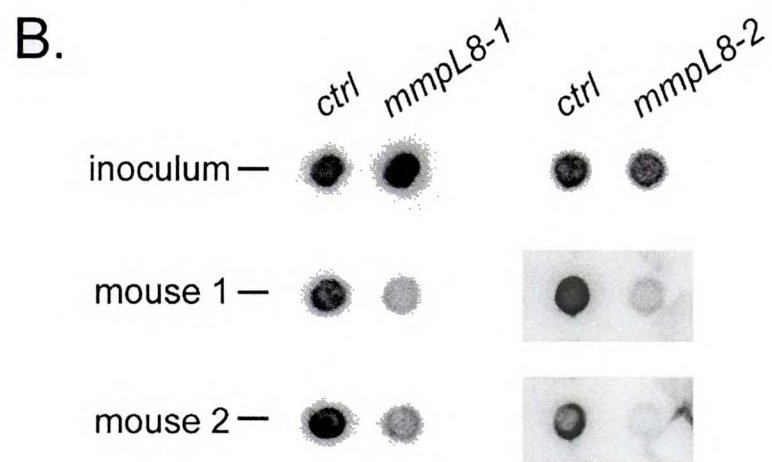
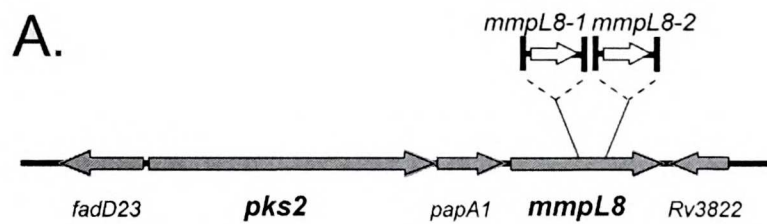
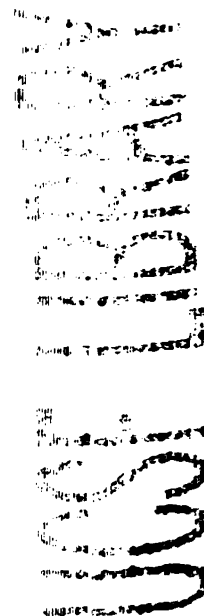
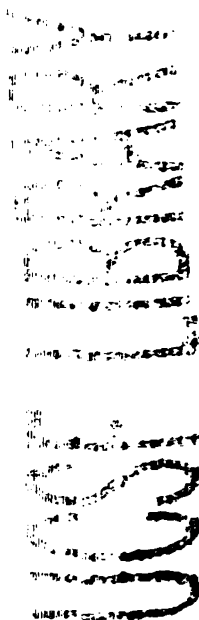




Figure 2. TLC analysis of lipids from *mmpL8* and *pks2* *M. tuberculosis* mutants.

(A) Cells were labeled with ^{14}C -propionate, and lipids were extracted and separated under two TLC solvent conditions. The first (top panel) resolves sulfolipid-1 (SL-1) whereas the second (bottom panel) resolves PDIM. Equal amounts of lipids were spotted onto both TLCs. The position of SL-1, Lipid 2, and the two forms of PDIM are noted. **(B)** Cells were labeled with $^{35}\text{S}\text{-SO}_4^{2-}$, and lipids were extracted and separated by TLC, as in Figure 1A, top panel. **(C)** Proposed structure of SL-1 as determined by Mougous, et al. and Goren, et al. (Goren, 1970; Goren et al., 1971; Mougous et al., 2002b).

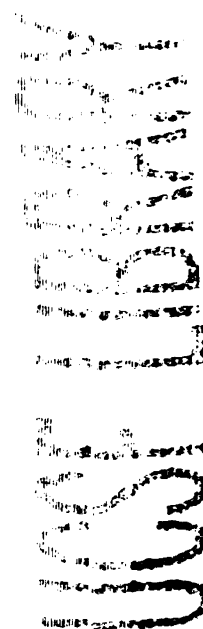


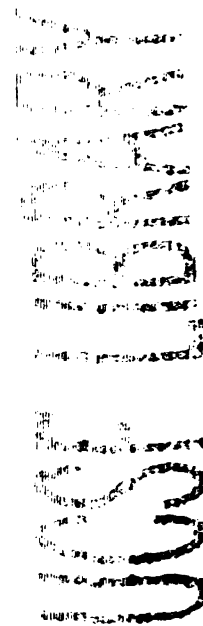
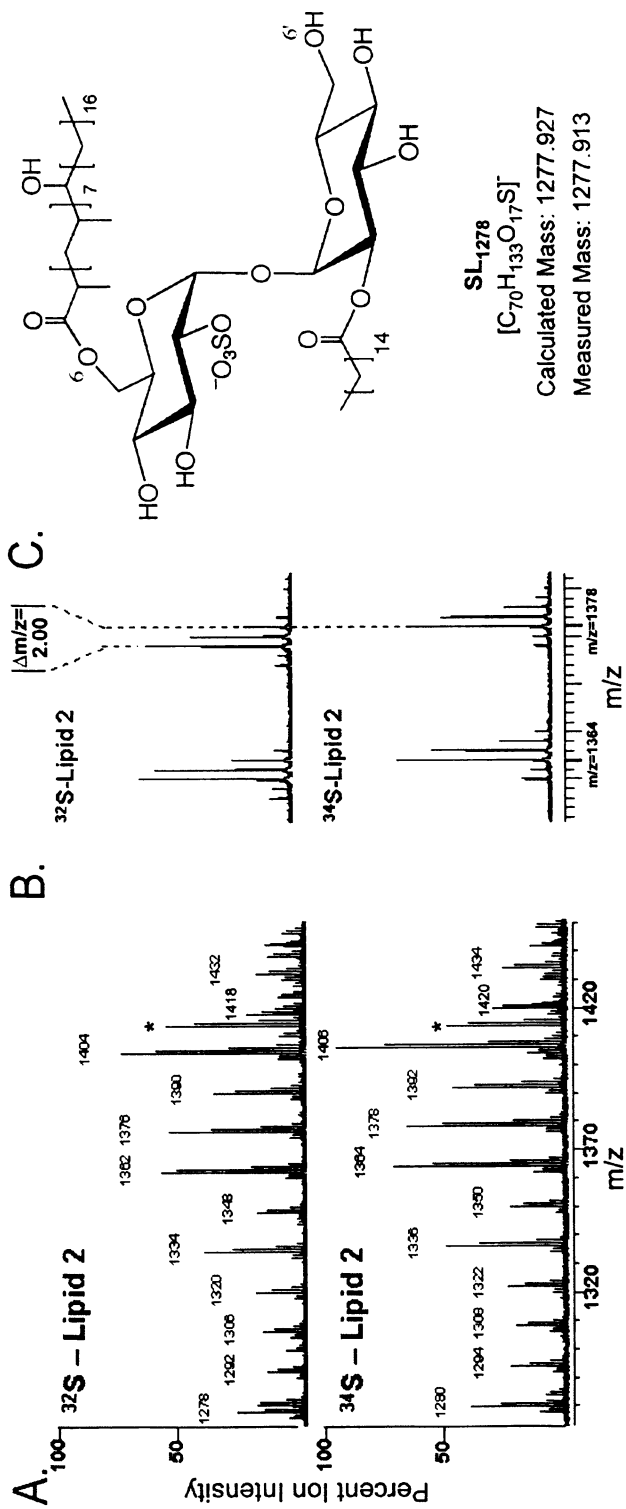


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Figure 3. Structural characterization of Lipid 2 by mass spectrometry.

(A) Cells were labeled with $^{32}\text{S-SO}_4^{2-}$ or $^{34}\text{S-SO}_4^{2-}$, Lipid 2 was partially purified by preparative TLC, and both lipid samples were analyzed by FT-ICR MS. Comparison of both spectra revealed a single group of species (m/z values denoted above each peak) that shifted by 2 mass units, indicating the presence of one sulfur atom in each molecule. An unrelated, sulfur-free compound within the spectra is marked with an asterisk. (B) A magnified view of the spectra from A. that shows two peaks that shifted when cells were labeled with $^{34}\text{S-SO}_4^{2-}$. (C) Proposed structure of the SL-related Lipid 2 species, SL_{1278} , as determined by exact mass analysis. The mass measured by MS differs from the calculated mass of the proposed structure by 11 ppm.



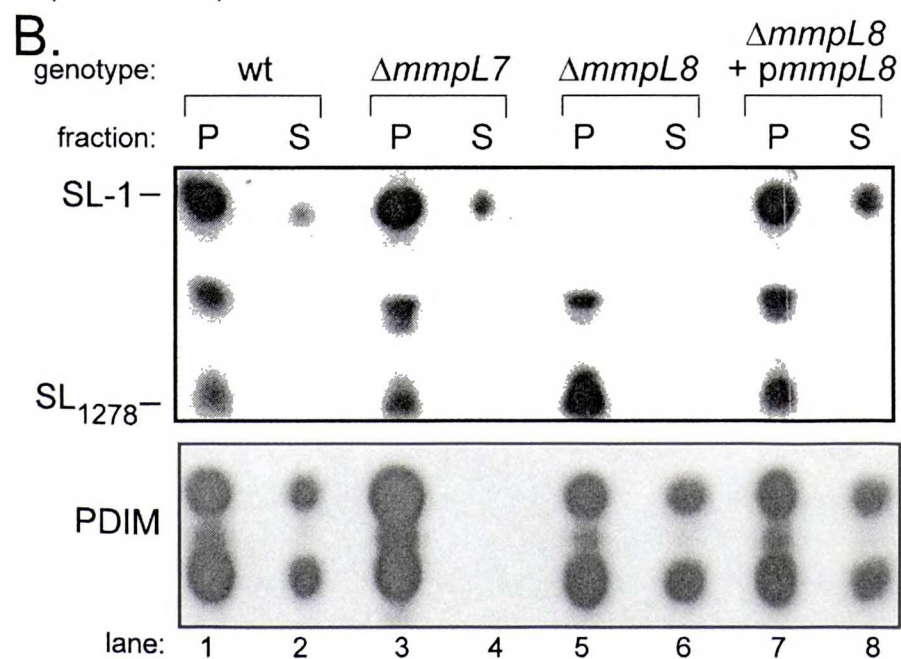
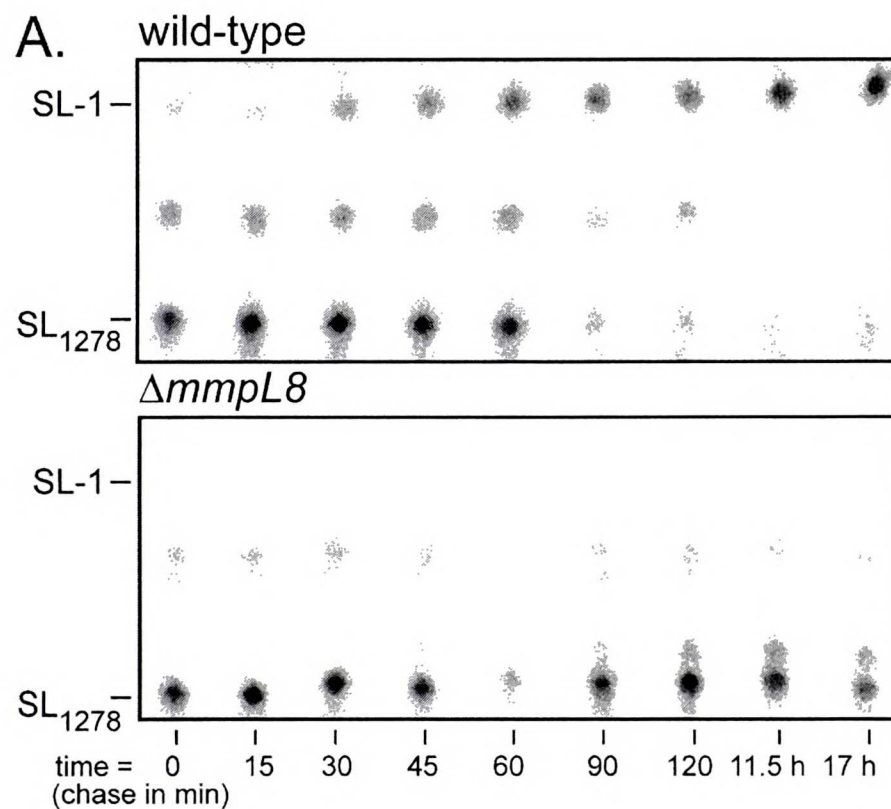


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Figure 4. SL₁₂₇₈ is a biosynthetic precursor of SL-1 and is not exposed on the mycobacterial cell surface.

(A) Wild-type (top panel) and $\Delta mmpL8$ mutant cells (bottom panel) were pulsed labeled with ¹⁴C-propionate for 30 minutes, washed to remove unincorporated label, and grown in liquid media. At the times indicated during the chase, cells were harvested, and lipids were extracted and analyzed by TLC. The $\Delta mmpL8$ sample at t=60 is underloaded, not deficient in SL₁₂₇₈. **(B)** Cells were labeled with ¹⁴C-propionate for 2 h, and surface-exposed lipids (S) were extracted using hexanes-decylamine and gentle sonication. Cell pellets (P) containing the lipids not extracted by this procedure were harvested by centrifugation and lipids from both fractions were extracted and analyzed by TLC.





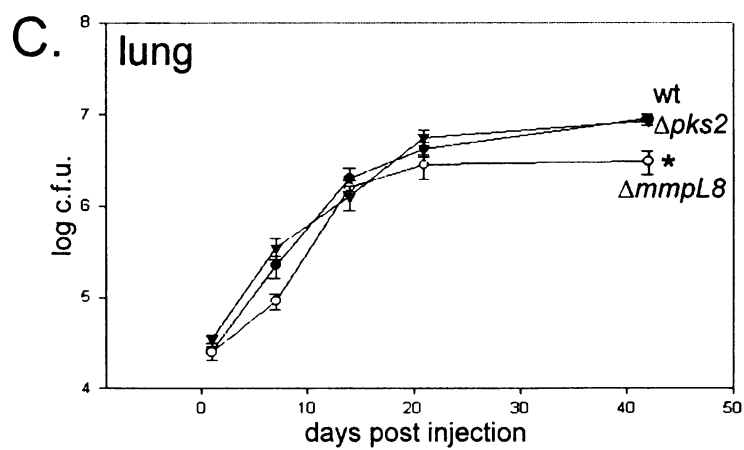
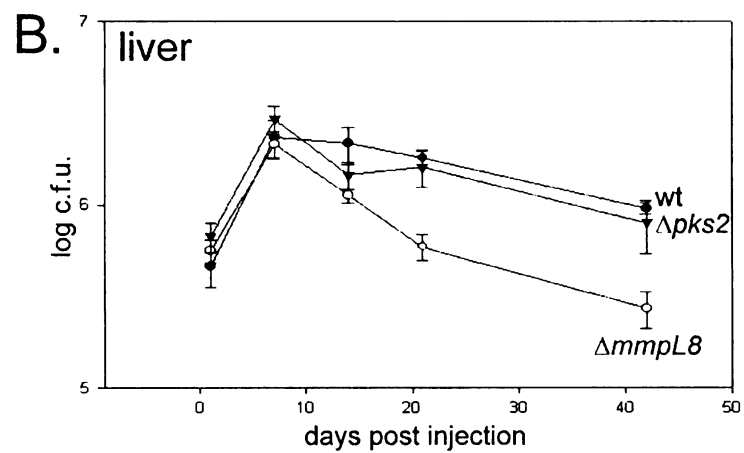
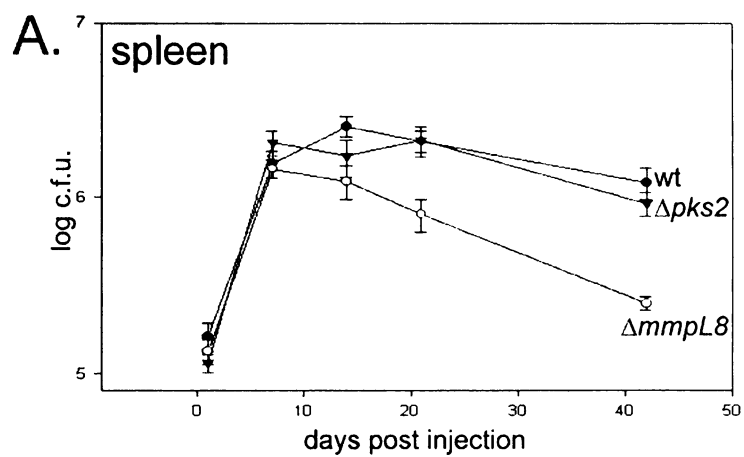
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Figure 5. *mmpL8*, but not *pks2*, is required for growth in a mouse model of tuberculosis.

C57/B6 mice were infected by tail-vein injection with $1-2 \times 10^6$ cells of wild-type (filled circles), $\Delta mmpL8$ (open circles), or $\Delta pks2$ (closed triangles) mutant *M. tuberculosis* cells. Growth in the spleen (A), liver (B), and lung (C) was monitored by harvesting organs at 1, 7, 14, 21, and 42 days post-infection, and bacillary loads were determined by plating dilutions on solid media. Each data point represents the average c.f.u. from 5 infected mice and error bars indicate the standard error of the means. The data shown here is from one experiment and a duplicative experiment gave nearly identical results. * = p-value of 0.003.





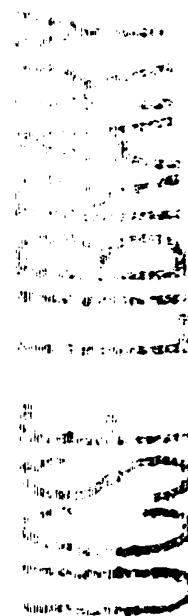


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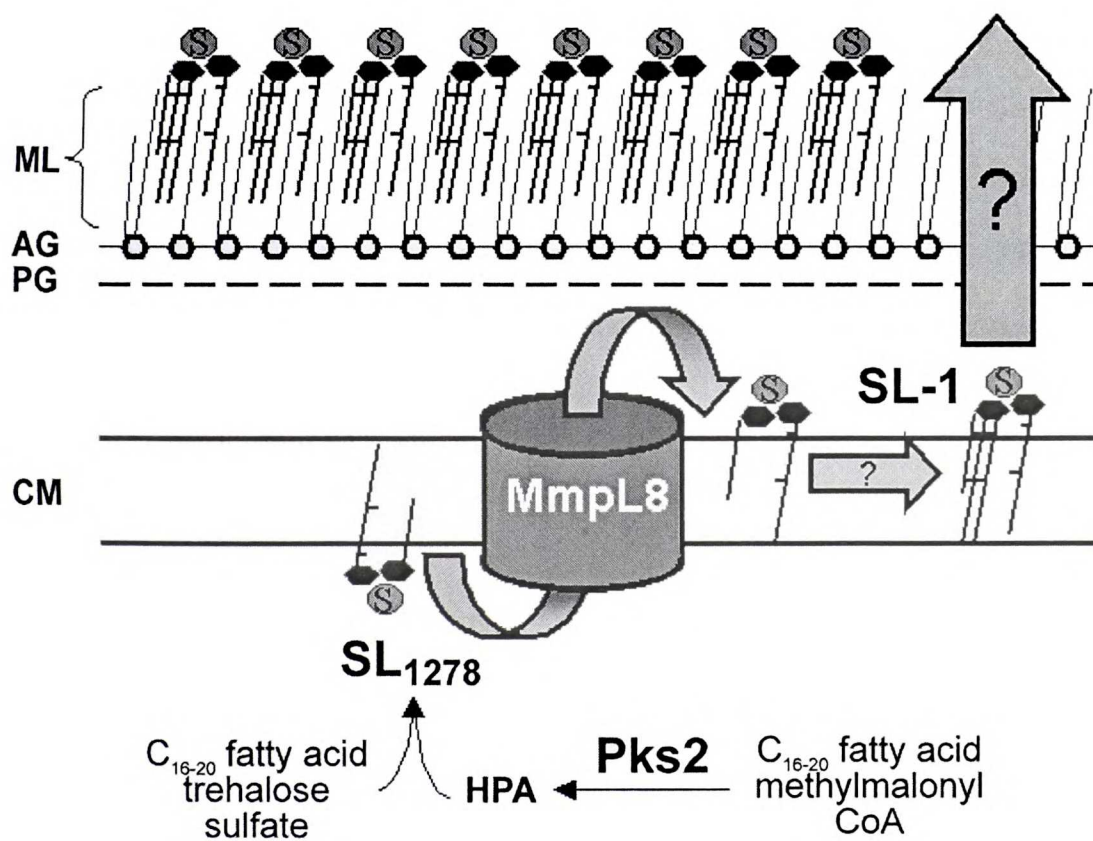
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Figure 6. Model of the role of MmpL8 in the SL-1 biosynthetic pathway.

See Discussion for details of the model. HPA= hydroxyphthioceranic acid, ML = mycolate layer, AG = arabinogalactan layer, PG = peptidoglycan layer, CM = cytoplasmic membrane.



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Chapter 3

**A Protein Secretion Pathway Critical for *Mycobacterium tuberculosis*
Virulence is Conserved and Functional in *Mycobacterium smegmatis***

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Abstract

The Snm protein secretion system is a critical determinant of *Mycobacterium tuberculosis* virulence. However, genes encoding components of this pathway are conserved among all mycobacteria, including the non-pathogenic saprophyte *Mycobacterium smegmatis*. We show that the Snm system is operational in *M. smegmatis* and that secretion of its homologous ESAT-6 and CFP-10 substrates is regulated by growth conditions. Importantly, we show that Snm secretion in *M. smegmatis* requires genes that are homologous to those required for secretion in *M. tuberculosis*. Using a gene knockout strategy in *M. smegmatis*, we have also discovered four new gene products that are essential for Snm secretion, including the serine protease Mycosin-1. Despite the evolutionary distance between *M. smegmatis* and *M. tuberculosis*, the *M. smegmatis* Snm system can secrete the *M. tuberculosis* ESAT-6 and CFP-10 proteins, suggesting that substrate recognition is also conserved between the two species. *M. smegmatis*, therefore, represents a powerful system to study the multi-component Snm secretory machine and to understand the role of this conserved system in mycobacterial biology.

Introduction

Throughout evolution, numerous bacterial pathogens have acquired specialized protein secretion pathways to deliver effector proteins to host cells (Christie, 2001; Lee and Schneewind, 2001; Thanassi and Hultgren, 2000).

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These pathways, which are distinct from the ubiquitous Sec pathway, are critical for mediating interactions during infection to allow pathogen survival in the hostile environment of the host. For example, the Type III secretion system is used by many pathogenic Gram-negative bacteria and requires the assembly of a complex translocon that transfers bacterial proteins directly from the bacterial cytoplasm into the eukaryotic host cell. Different bacterial pathogens secrete distinct sets of Type III effectors, each with specific activities tailored to meet the individual needs of the bacterium (Lee and Schneewind, 2001). The Type III secretion system and other Sec-independent secretion pathways in Gram-negative bacteria are well-studied, but analogous pathways in Gram-positive pathogens are poorly understood (Tjalsma et al., 2004).

Initial clues of the existence of a Sec-independent protein secretion pathway in *M. tuberculosis* came from studies of the immunodominant protein ESAT-6 (early secretory antigenic target, 6kDa). This protein, along with its binding partner CFP-10 (culture filtrate protein, 10kDa), is efficiently secreted from *M. tuberculosis* cells but lacks N-terminal signal sequences that would target its secretion through the Sec pathway (Skjot et al., 2000; Sonnenberg and Belisle, 1997; Sorensen et al., 1995). The genes encoding ESAT-6 and CFP-10, *esxA* and *esxB*, are co-transcribed in a two-gene operon (Berthet et al., 1998). The predicted function of the genes surrounding this operon led to the hypothesis that neighboring genes may be important for ESAT-6 and CFP-10 secretion (Cole et al., 1998; Gey Van Pittius et al., 2001; Pallen, 2002; Tekai et al., 1999). Furthermore, this region of the genome, known as the

RD1 locus for Region of Difference 1, is notable as the *esxA/esxB* operon and several flanking genes have been deleted from *M. bovis* during the repeated passage that led to the attenuated BCG vaccine strain. Attempts to rescue ESAT-6 and CFP-10 export in BCG revealed that only a cosmid containing the entire genomic locus that surrounds the *esxA/esxB* operon could restore ESAT-6 and CFP-10 secretion (Pym et al., 2003). These results suggest that ESAT-6 and CFP-10 secretion requires genes both upstream and downstream of the *esxA/esxB* operon, although the specific genes required for this pathway remained obscure.

Recent genetic studies have demonstrated that individual conserved genes within the RD1 region in *M. tuberculosis* encode for an alternative protein secretion pathway, termed the *Snm* (secretion in mycobacteria) pathway (Guinn et al., 2004; Hsu et al., 2003; Stanley et al., 2003). A screen seeking mutants specifically defective for growth in the mouse model of infection isolated a class of mutants with transposon insertions clustered near the *esxA/esxB* operon (Stanley et al., 2003). Strikingly, these mutants were capable of synthesizing ESAT-6 and CFP-10, yet were unable to secrete them into the extracellular milieu. The transposons disrupted three genes, *snm1* (*Rv3870*), *snm2* (*Rv3871*), and *snm4* (*Rv3877*) that encode for two ATPases and a 12 transmembrane domain protein, respectively. In similar studies, a proline-rich predicted chromosome-partitioning ATPase (*Rv3876* or *snm3*) was also shown to be required for ESAT-6 and CFP-10 secretion (Guinn et al., 2004).

Evidence that the Snm proteins form a secretory apparatus to assist in ESAT-6 and CFP-10 secretion came from biochemical and yeast two-hybrid studies. Purified ESAT-6 and CFP-10 formed a stable 1:1 dimer, and yeast two-hybrid results have confirmed this strong interaction (Okkels and Andersen, 2004; Renshaw et al., 2002; Stanley et al., 2003). Furthermore, physical interactions were detected between individual components of the Snm machinery (Snm1 and Snm2) and between the machinery and its secretion substrates (Snm2 and CFP-10) (Stanley et al., 2003). These findings led to our working model that Snm2 recruits the ESAT-6:CFP-10 dimer to the cytoplasmic membrane via its interactions with CFP-10 and Snm1, which has three predicted transmembrane domains. Consistent with the role of ATPases in other protein secretion pathways (Holroyd and Erdmann, 2001; Ye et al., 2001), concerted ATP hydrolysis by Snm1 and Snm2 would promote ESAT-6:CFP-10 translocation through the secretory pore consisting of Snm4 (see Figure 5).

Bioinformatic analysis of the *M. tuberculosis* genome has revealed several interesting features of the ESAT-6 and CFP-10 secretion pathway (Cole et al., 1998; Gey Van Pittius et al., 2001; Pallen, 2002; Pallen et al., 2003; Tekai et al., 1999). First, the genes that encode ESAT-6 and CFP-10 have duplicated numerous times in the *M. tuberculosis* genome, resulting in 10 additional operons homologous to *esxA/esxB*. Interestingly, at five of the eleven loci, a conserved set of ten genes, including *snm1-4* homologues, are syntenic with the *esx* operon. The proximity of the six other genes within these

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esx loci suggests that they encode for additional Snm proteins that function during ESAT-6 and CFP-10 secretion.

Interestingly, the Snm pathway and its esx substrates are not unique to mycobacterial human pathogens, such as *M. tuberculosis* and *M. leprae*. Transposon mutagenesis in the ectotherm pathogen, *Mycobacterium marinum*, uncovered a homologous Snm pathway that was critical for host cell cytolysis and virulence (Gao et al., 2004). Furthermore, the conserved esx operon and several of the surrounding *snm* genes have been identified in other Gram-positive bacteria, including *Bacillus subtilis* and *Corynebacterium diphtheriae* (Pallen, 2002). However, only mycobacteria possess the full set of conserved genes from *M. tuberculosis* that surround the esx operons. Interestingly, *Mycobacterium smegmatis*, a soil-dwelling, distant relative of *M. tuberculosis*, contains clear homologues of five of the eleven esx loci in *M. tuberculosis*, including the *esxA/esxB* locus and all the surrounding known or putative *snm* genes (Gey Van Pittius et al., 2001, see Fig. 1).

As *M. smegmatis* possesses a highly homologous *esxA/esxB* genomic locus and has many experimental advantages compared to *M. tuberculosis* (biochemically and genetically tractable, non-pathogenic, rapid growth), we sought to utilize this organism in order to understand the mechanism and role of the Snm pathway. In this study, we show that the functionally conserved *M. smegmatis* Snm pathway secretes its homologous ESAT-6 and CFP-10 substrates (which we designate SmESAT-6 and SmCFP-10) and is regulated by growth conditions. By creating in-frame deletions of the genes surrounding

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the *esxA/esxB* locus in *M. smegmatis*, we have also identified four new *Snm* proteins that are required for SmESAT-6 and SmCFP-10 secretion.

Results

Similarity of *esxA/esxB* loci in *M. smegmatis* and *M. tuberculosis*.

The *M. smegmatis* genomic sequence was obtained from TIGR (<http://www.tigr.org/tdb/ufmg/>), and our preliminary annotation revealed that the *esxA/esxB* genomic locus is highly conserved between *M. smegmatis* and *M. tuberculosis* (Figure 1A). The loci are organized in a similar fashion, and *M. smegmatis* possesses strong homologues for all of the known and putative *snm* genes (Gey Van Pittius et al., 2001). There are, however, some interesting differences between the two loci. For example, BLAST searches of a 4459-bp region 5' of the *Sm3881* gene revealed three genes that encode for putative transposases that are absent in *M. tuberculosis*. It is possible that these transposases and their associated transposons may have mediated the extensive duplication and/or horizontal gene transfer of the *esx* locus. Furthermore, the *M. smegmatis sm3* homologue encodes for a product lacking the proline-rich N-terminus of its counterpart in *M. tuberculosis*. Despite these differences, however, the overall conservation of the *esxA/esxB* locus suggests that SmESAT-6 and SmCFP-10 may be secreted by *M. smegmatis* (Gey Van Pittius et al., 2001).

Handwritten musical notation for the song "The Rose Tree". The notation is written on a single staff with a treble clef. The melody is simple and consists of several measures of music, including eighth and sixteenth notes, and rests. The lyrics "The Rose Tree" are written below the staff.

Analysis of SmESAT-6 and SmCFP-10 secretion under various growth conditions in *M. smegmatis*.

To test this hypothesis, we examined SmESAT-6 and SmCFP-10 secretion into the culture media during growth in standard 7H9 liquid media. Wild-type *M. smegmatis* was grown to log-phase, cells were removed by centrifugation, and the supernatant was filtered and concentrated into the short-term culture filtrate (STCF) fraction. Proteins were extracted from the cell-associated and STCF fractions, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blot with polyclonal antibodies raised against *M. smegmatis* SmESAT-6 and *M. tuberculosis* SmCFP-10. As shown in Figure 1B, when cultured in 7H9 media, SmESAT-6 and SmCFP-10 are present in the cell-associated whole cell lysate (P) fraction, but absent from the STCF (S) fraction (lanes 1 & 2). Both proteins have apparent molecular masses similar to their homologues in *M. tuberculosis* (M.W._{ESAT-6} = 8.4 kDa; M.W._{CFP-10} = 11.5 kDa, Mattow et al., 2001; Rosenkrands et al., 2000). Thus, although SmESAT-6 and SmCFP-10 were clearly expressed by *M. smegmatis* cells grown in 7H9 media, they did not appear to be secreted.



For technical reasons, STCF fractions are typically prepared from *M. tuberculosis* cultures grown in Sauton's media, and we reasoned that SmESAT-6 and SmCFP-10 secretion in *M. smegmatis* may be inhibited by the growth conditions present in 7H9. Therefore, to mimic more closely the *in vitro* culturing conditions used for *M. tuberculosis* (Guinn et al., 2004; Sorensen et

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al., 1995; Stanley et al., 2003), we also prepared STCF fractions from *M. smegmatis* cultured in Sauton's media. Strikingly, both SmESAT-6 and SmCFP-10 are secreted into the STCF fraction when grown in this media (Figure 1B, lanes 3 & 4). In order to determine the temporal pattern of SmESAT-6 secretion, we cultured a large volume of wild-type *M. smegmatis* and collected whole-cell and STCF fractions at various times. As shown in Figure 6, SmESAT-6 was present in the pellet at all time points tested, but it accumulated in the STCF fraction only at later time points (T=16 & 20 hours). Cell autolysis did not contribute significantly to the protein profiles in the STCF fractions as two intracellular proteins, KatG and GroEL, which accumulate extracellularly under prolonged growth conditions in 7H9 and Sauton's media (Recht et al., 2000; Tullius et al., 2001, data not shown) were exclusively cell-associated at the time all STCF fractions were harvested (Figure 1B, lower panels). To show that our antibodies specifically recognized SmESAT-6 and SmCFP-10, we analyzed extracts prepared from an $\Delta esxB$ mutant (see below), as deletion of *esxB* in *M. tuberculosis* abolishes both SmESAT-6 and SmCFP-10 expression (Stanley et al., 2003). As expected, the bands corresponding to SmESAT-6 and SmCFP-10 were missing in extracts from the *M. smegmatis* $\Delta esxB$ mutant (Figure 1B, lanes 5-8). STCF protein profiles were qualitatively similar between 7H9 and Sauton's media (data not shown), suggesting that this media-dependence is specific for SmESAT-6 and SmCFP-10 export. Based on these findings, we conclude that SmESAT-6 and SmCFP-10 secretion into the

[illegible]

STCF fraction, but not expression, is dependent on the growth media; therefore, we cultured *M. smegmatis* in Sauton's media for the remainder of these studies.

Construction of *snm* and *esx* in-frame deletion strains.

To test if the *Snm* secretion pathway functions similarly in both *M. smegmatis* and *M. tuberculosis*, we sought to make *M. smegmatis* strains that lacked each of the homologues of *snm1*, *snm2*, *snm4*, *esxA*, and *esxB* genes. In-frame deletion constructs were created by PCR and inserted into a vector that contained the *sacB* counterselectable marker. In the first of a 2-step homologous recombination procedure, wild-type *M. smegmatis* was transformed using these constructs to yield merodiploid strains that contained the *sacB* gene flanked by both the wild-type and mutated copy of the targeted gene in the genome (Pavelka and Jacobs, 1999, Figure 2A, see Table 1 for strain table). These strains were then grown in liquid culture and subsequently plated on solid media containing sucrose to select for cells in which the *sacB* gene had been deleted. Individual colonies were isolated and tested for proper deletion of the targeted gene by PCR. In all cases, the distribution of recombination events that led either to retention of the wild-type or the deletion allele was nearly 50:50, demonstrating that none of the genes are essential for viability (data not shown). Furthermore, these mutations had no effect on colony morphology (see Figure 7) or bacterial growth as the growth rate of each mutant strain was identical to the wild-type strain (data not shown). Accurate deletion of each gene was confirmed by Southern blot analysis, which revealed

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the expected patterns for the deletion strains (Figure 2B, see Table 2 for details), and sequence analysis of the integrated DNA confirmed that the deletions were in-frame and that no additional mutations arose during the construction.

Conservation of Snm machinery between *M. tuberculosis* and *M. smegmatis*.

As predicted, Western blot analysis of proteins extracted from the *snm1*, *snm2*, and *snm4* deletion strains showed that SmESAT-6 and SmCFP-10 secretion, but not synthesis, is blocked in these mutants (Figure 3A, lanes 1-6, 11-12). Furthermore, SmESAT-6 and SmCFP-10 are absent in their respective deletion strains (Figure 3A, lanes 7-10). In *M. tuberculosis*, the loss of either *esxA* or *esxB* alone abolishes the expression of both ESAT-6 and CFP-10, though polar effects cannot be excluded (Stanley et al., 2003). In-frame deletions of the *esxA* and *esxB* homologues in *M. smegmatis* show a similar relationship: *esxB* is required for the expression of SmESAT-6 and *esxA* is required for full expression of SmCFP-10. While low levels of SmCFP-10 are apparent in the lysate fraction of the *esxA* mutant (Figure 3A, lane 9), the expression of both homologues is required for substantial expression of both SmESAT-6 and SmCFP-10. Silver staining of the STCF fractions from the mutant strains revealed indistinguishable secretion profiles compared to the wild-type strain, which suggests that these mutations do not cause gross secretion defects (Figure 3A, right panel). In sum, these results clearly



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demonstrate that a functional *Snm* secretion apparatus exists in *M. smegmatis* and requires the homologous components to those required in *M. tuberculosis*.

Identification of novel *snm* mutants.

Since *M. smegmatis* secretes SmESAT-6 and SmCFP-10 in an *Snm*-dependent fashion, we sought to utilize this powerful model system to discover new components of the *Snm* pathway. To this end, we generated in-frame deletions of the 5 putative *snm* genes from the *esxA/esxB* locus using the same 2-step strategy described above (Figure 2). Strikingly, Western blot analysis of protein extracts harvested from these deletion strains revealed that four of the five genes, *Sm3866*, *Sm3869*, *Sm3882c*, and *mycP1* (*Sm3883c*) are required for SmESAT-6 and SmCFP-10 secretion (Figure 3B). Interestingly, *Sm3883c* encodes for a previously identified membrane-anchored serine protease termed Mycosin-1 (Brown et al., 2000; Dave et al., 2002). *Sm3866* shares limited homology with ABC-ATPases involved in Type I secretion (Thompson et al., 1998), and *Sm3869* encodes for a membrane protein with predicted ATP/GTP binding sites. Finally, *Sm3882c* is likely in an operon with *mycP1* and encodes for an uncharacterized membrane protein. We propose that the previously uncharacterized *snm* genes be named *snm5* (*Sm3866*), *snm6* (*Sm3869*), and *snm7* (*Sm3882c*). Interestingly, a predicted AAA-ATPase, *Sm3868*, is not required for SmESAT-6 and SmCFP-10 secretion despite its high sequence conservation (76% identity) between *M. smegmatis* and *M. tuberculosis*. In summary, these results identify four additional genes that are required for the

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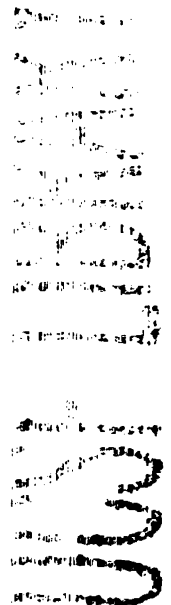
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Sm pathway, and it suggests that a large, multi-component secretion apparatus functions during the process of SmESAT-6 and SmCFP-10 secretion in *M. smegmatis*.

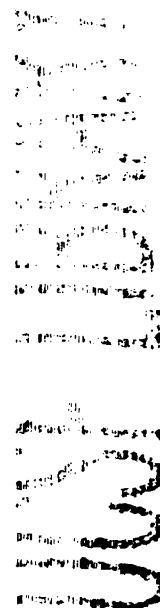
Complementation of novel *snm* and *esx* mutants.

In order to confirm that our newly discovered *snm* genes are indeed functioning during SmESAT-6 and SmCFP-10 secretion, we sought to complement their secretion defects. Constitutive expression using the *M. tuberculosis groEL2* promoter of tagged Sm3866 and Sm3869 in the appropriate deletion strain fully restored SmESAT-6 and SmCFP-10 secretion compared to the deletion strains containing the empty vector (Figure 4A, lanes 1-10). Expression of Sm3882c and Mycosin-1 from this promoter caused cell toxicity (data not shown). Therefore, as both genes are likely in an operon (see Figure 1), we engineered a new complementation plasmid in which the constitutive *groEL2* promoter was replaced with the 505 bp predicted *mycP1* promoter region. As shown in Figure 4B, lanes 11-18, these complemented *Sm3882c* and *mycP1* mutants secrete SmESAT-6 and SmCFP-10 to approximately wild-type levels. Furthermore, cell autolysis induced by exogenous gene expression is not responsible for the complemented phenotype as KatG and GroEL are both exclusively cell-associated in all strains (Figure 4A-C, lower panels). These results prove that Sm3866, Sm3869, Sm3882c, and Mycosin-1 function during SmESAT-6 and SmCFP-10 secretion.



To rule out the possibility of polar effects in the $\Delta esxA$ and $\Delta esxB$ mutant strains, we used an episomal plasmid containing the appropriate untagged gene driven by the *groEL2* promoter for complementation analysis. Expression of SmCFP-10 and SmESAT-6 in their respective deletion strain rescued the synthesis and secretion of both SmCFP-10 and SmESAT-6 compared to vector-transformed knock-out strains (Figure 4B, lanes 1-8). Interestingly, C-terminally tagged versions of SmESAT-6 and SmCFP-10 were inefficiently secreted, although the reason for this is unclear (data not shown).

We next investigated if the *M. tuberculosis* ESAT-6 and CFP-10 homologues could be secreted by the *M. smegmatis* system (Figure 4C). Importantly, the *M. tuberculosis* ESAT-6 monoclonal antibody, which readily detects secreted *M. tuberculosis* ESAT-6 (Stanley et al., 2003), does not recognize endogenous *M. smegmatis* SmESAT-6 (lanes 1 & 2). Introduction of an episomal plasmid, in which expression of *M. tuberculosis* *esxA* and *esxB* is driven by the synthetic *mop* promoter (Guinn et al., 2004), into $\Delta esxA$ cells resulted in synthesis and secretion of the *M. tuberculosis* homologues (lanes 3 & 4). This result demonstrates that substrate recognition is similar between the two species. Moreover, our results demonstrate a high degree of functional conservation of the *Snm* secretory apparatus between *M. smegmatis* and *M. tuberculosis*.



Discussion

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In this study, we have presented the first functional characterization of the Snm alternative protein secretion pathway in a non-pathogenic bacterium *M. smegmatis*. *M. smegmatis* possesses an *esx/snm* locus homologous to that from *M. tuberculosis* and secretes SmESAT-6 and SmCFP-10 into the extracellular milieu. Our genetic analysis of this locus revealed that SmESAT-6 and SmCFP-10 secretion strictly requires homologues of *snm1*, *snm2*, and *snm4*, which are required for secretion in *M. tuberculosis*. Furthermore, the *M. smegmatis* Snm machinery secretes the *M. tuberculosis* ESAT-6 and CFP-10 homologues. Therefore, the Snm system appears to function analogously in both organisms, providing an opportunity to study this pathway in a more tractable organism.

Indeed, by studying the Snm pathway in *M. smegmatis* we have identified four additional *snm* genes and contributed to our working model of the Snm alternative protein secretion pathway (Figure 5). Of particular interest is our finding that the serine protease, Mycosin-1, is required for Snm secretion. Given its predicted periplasmic location, it may act like the Sec-pathway signal peptidase which removes N-terminal signal sequences upon secretion (Paetzel et al., 2002). However, as SmESAT-6 and SmCFP-10 show no obvious size change upon export, we hypothesize that Mycosin-1 may act as a regulator of Snm secretion. For example, its proteolytic activity may either degrade an Snm inhibitor or activate an Snm protein, perhaps in the cell wall (Brown et al., 2000; Dave et al., 2002). Given the functional conservation between the Snm pathway in *M. smegmatis* and *M. tuberculosis*, it is likely that *mycP1* and *snm5*-

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7 will also be required for ESAT-6 and CFP-10 secretion in *M. tuberculosis*. Of course, verification will require mutation of these individual genes in *M. tuberculosis*. Importantly, we were able to complement all of the newly found *snm* mutants with individual genes, confirming that the proteins function during SmESAT-6 and SmCFP-10 secretion in *M. smegmatis*.

An unexpected finding of these experiments was that SmESAT-6 and SmCFP-10 are secreted during growth in Sauton's media but not in 7H9. Because there are numerous differences between Sauton's and 7H9 media, it seems likely that the Snm system is responsive to one or more components of the growth media. In order to determine if SmESAT-6 secretion regulation might reflect a dominant property of either Sauton's or 7H9 media, we performed mixing experiments, in which we mixed equal parts of Sauton's and 7H9 media and performed the SmESAT-6 secretion assay. As shown in Figure 8, SmESAT-6 is not secreted into the mixed media STCF fraction, which is consistent with 7H9 media containing one or more components which block SmESAT-6 secretion. Unfortunately, adding each component singly to Sauton's media did not block secretion, and conversely, removing each component singly from 7H9 media did not enable secretion (see Table 3 for all media types tested). We, therefore, hypothesize that *M. smegmatis* responds to multiple constituents in 7H9 media to regulate the Snm pathway. Our efforts to understand this potential regulation are ongoing; however, this is the first suggestion that the Snm pathway may be regulated and reveals a potential similarity with Sec-independent secretion pathways from Gram-negative

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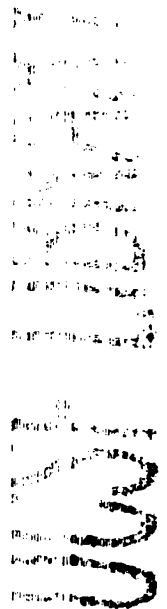
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bacteria, which also require strict media conditions in order for active protein export (Dasgupta et al., 2004; Rietsch et al., 2004).

The unique mycobacterial cell wall represents an enormous barrier to protein secretion, and it remains unknown how ESAT-6 and CFP-10 are translocated across all of the layers of the thick mycobacterial cell wall. In addition to the peptidoglycan layer, mycobacteria possess hydrophobic long chain mycolic acids esterified to a polymeric arabinogalactan layer (Figure 5). There are at least two models for how ESAT-6 and CFP-10 are exported across the cell wall. First, the Snm system may translocate these proteins only across the cytoplasmic membrane, leaving them to diffuse across the cell wall, perhaps through porins or passive processes (Niederweis, 2003; Pym et al., 2002). Alternatively the Snm system may function analogously to Type III secretion and provide a continuous conduit through which ESAT-6 and CFP-10 are translocated from the bacterial cytosol directly across all cell wall layers.

Finally, why might the Snm system be conserved in *M. smegmatis* yet absolutely critical for *M. tuberculosis* virulence? We envision several possibilities. First, the pathway may play an identical and fundamental role in both organisms, perhaps in cell-to-cell communication. In support of this, Flint, et al. (Flint et al., 2004) recently reported that *M. smegmatis snm* mutants display increased conjugation efficiency and present evidence that Snm secretion suppresses conjugation *in trans*. Although conjugal transfer has not been detected in *M. tuberculosis*, it is plausible that both organisms may utilize ESAT-6 and CFP-10 as density-dependent signals to coordinate cell activities.



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Finally, in accordance with their different environmental niches, each species may secrete additional substrates besides ESAT-6 and CFP-10. Thus, the Snm pathway, like other Sec-independent pathways, may be modular, allowing substrates to evolve for the particular needs of each organism. Thus, studying the Snm pathway in *M. smegmatis* will help delineate not only the mechanism of Snm secretion, but perhaps uncover the evolutionarily conserved role of the Snm pathway.

Materials and Methods

Protein preparation and analysis.

M. smegmatis was inoculated from a single colony and grown to mid-log phase ($OD_{600}=0.6-0.8$) in 7H9 media supplemented with 0.05% Tween-80 (Fisher Scientific). Cells were washed and inoculated in 7H9 or Sauton's media supplemented with 0.05% Tween-80. Upon reaching mid-log phase ($OD_{600}=0.6-0.8$), cells were washed and inoculated in 7H9 or Sauton's media without Tween-80 and grown to an $OD_{600}=0.8$. Cells were harvested by centrifugation and the cultured media supernatant was filtered through a $0.22\mu m$ filter and concentrated using an Amicon Ultra-15 (5,000 MWCO, Millipore). For complementation experiments, media was supplemented with kanamycin ($20\mu g/ml$, Fisher Scientific). Whole cell lysates and short-term culture filtrate (corresponding to 5ml original culture volume) fractions were precipitated with TCA and analyzed by SDS-PAGE using 4-20% Tris-HCl

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Criterion gels (Biorad). For immunoblotting of *M. smegmatis* SmESAT-6, polyclonal antibodies were raised against the N-terminal 20-aa plus a C-terminal cysteine (Covance Research Products, Inc.), and the antibody was affinity purified using the Sulfolink kit (Pierce Biotechnology). The antibodies against CFP-10 (K8493) and GroEL (HAT5) were kind gifts of Dr. P. Anderson. The antibody against KatG was a kind gift of Dr. S. Cole.

Construction of *M. smegmatis* Deletion Strains and Southern Analysis.

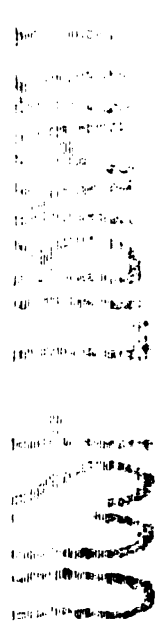
To generate deletion strains, the 5' and 3' 800-bp flanks surrounding each gene were amplified by PCR, sequence analyzed, and introduced into pjsc232, creating an *Afl*III site at the junction between the flanks. This strategy created an in-frame gene deletion which encodes a truncated gene product. See Table 2 for precise truncated sizes of each deleted gene. These plasmids were introduced as described (Hinds et al., 1999), and integrants were selected by growing on 7H10 agar containing kanamycin. Single colonies were picked and grown to late-log phase in liquid 7H9 media without kanamycin, and recombinants were selected by growing on 7H10 agar with 5% sucrose. Deletion generation was confirmed by PCR and Southern analysis. See Table 1 for strain table and Table 2 for more details of Southern analysis.

Construction of *snm* and *esx* complementation plasmids.

Complementation vectors were derived from pMV261.kan, a high-copy episomal plasmid in which transcription is driven by the constitutive *groEL2*

promoter (Stover et al., 1991). C-terminal 2xHA (pMJ13) or myc (pMJ31) tags were created by PCR and ligated into pMV261.kan. Each of the *snm* and *esx* genes were amplified by PCR from wild-type *M. smegmatis* genomic DNA, sequenced, and introduced into pMJ13 or pMJ31 creating C-terminally tagged constructs.

In order to complement the *Sm3882c* and *mycP1* mutant secretion defect, the *groEL2* promoter in pMJ31 was replaced with the PCR-amplified 505 bp predicted promoter immediately upstream of the *mycP1* gene. To generate *esxA* and *esxB* complementation constructs, a stop codon was introduced immediately after the open reading frame using the QuikChange site-directed mutagenesis kit (Stratagene). The pMH406 *M. tuberculosis esxA/B* complementation plasmid was a kind gift of Dr. D. Sherman. See Table 1 for strain and plasmid table.



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Table 1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics
<i>mc</i> ² 155	wild-type
SCM17	Δ <i>Sm3866</i> (<i>snm5</i>)
SCM25	Δ <i>Sm3868</i>
SCM29	Δ <i>Sm3869</i> (<i>snm6</i>)
SCM30	Δ <i>snm1</i> (<i>Sm3870</i>)
SCM26	Δ <i>snm2</i> (<i>Sm3871</i>)
SCM27	Δ <i>esxB</i> (<i>cfp-10</i> ; <i>Sm3874</i>)
SCM31	Δ <i>esxA</i> (<i>esat-6</i> ; <i>Sm3875</i>)
SCM32	Δ <i>snm4</i> (<i>Sm3877</i>)
SCM28	Δ <i>Sm3882c</i> (<i>snm7</i>)
SCM21	Δ <i>mycP1</i> (<i>snm8</i> ; <i>Sm3883c</i>)
SCM67	wild-type + pMJ31
SCM72	Δ <i>Sm3866</i> + pMJ31
SCM41	Δ <i>Sm3866</i> + pSEC43 (<i>Sm3866-myc</i>)
SCM73	Δ <i>Sm3869</i> + pMJ31
SCM45	Δ <i>Sm3869</i> + pSEC47 (<i>Sm3869-myc</i>)
SCM79	Δ <i>Sm3882c</i> + pMJ31
SCM120	Δ <i>Sm3882c</i> + pSEC80 (<i>Sm3882c-2xHA</i>)
SCM80	Δ <i>mycP1</i> + pMJ31
SCM122	Δ <i>mycP1</i> + pSEC82 (<i>mycP1-2xHA</i>)
SCM76	Δ <i>esxB</i> + pMJ31
SCM141	Δ <i>esxB</i> + pSEC108 (<i>esxB</i>)
SCM77	Δ <i>esxA</i> + pMJ31
SCM142	Δ <i>esxA</i> + pSEC109 (<i>esxA</i>)
SCM143	Δ <i>esxA</i> + pMH406 (<i>M.tb</i> <i>esxA/B</i>)
Plasmids	
pMJ13	<i>groEL2</i> promoter, C-terminal in-frame 2xHA tag, Kan ^R
pMJ31	<i>groEL2</i> promoter, C-terminal in-frame <i>myc</i> tag, Kan ^R
pSEC43	<i>groEL2</i> promoter, <i>Sm3866-myc</i> , Kan ^R
pSEC47	<i>groEL2</i> promoter, <i>Sm3869-myc</i> , Kan ^R
pSEC108	<i>groEL2</i> promoter, untagged <i>esxB</i> , Kan ^R
pSEC109	<i>groEL2</i> promoter, untagged <i>esxA</i> , Kan ^R
pSEC72	<i>mycP1</i> promoter, C-terminal in-frame 2xHA tag, Kan ^R
pSEC80	<i>mycP1</i> promoter, <i>Sm3882c-2xHA</i> , Kan ^R
pSEC82	<i>mycP1</i> promoter, <i>mycP1-2xHA</i> , Kan ^R
pMH406	<i>mop</i> promoter, <i>M.tb</i> <i>esxA/B</i> , Kan ^R (Guinn, Hickey et al 2004)



Table 2. Details of Southern blot analysis of *M. smegmatis* mutant strains

Strain	Enzyme(s)	Probe	wild-type gene expected size (in bp)	Δ gene expected size (in bp)	wild-type gene product size (in aa)	Δ gene product size (in aa)
Δ Sm3866 (<i>snm5</i>)	AflII, SphI	847-bp 3' flank	4076	2374	294	31
Δ Sm3868	AflII, SphI	831-bp 5' flank	4076	2311	574	38
Δ Sm3869 (<i>snm6</i>)	AflII, BstBI	886-bp 5' flank	17751	5518	479	29
Δ snm1 (<i>Sm3870</i>)	AflII, NcoI	871-bp 3' flank	3822	1546	742	32
Δ snm2 (<i>Sm3871</i>)	AflII, SphI	841-bp 3' flank	3879	1316	593	20
Δ esxB (<i>cfp-10</i>)	AflII, SphI	820-bp 3' flank	4619	3741	100	13
Δ esxA (<i>esat-6</i>)	AflII, SphI	842-bp 3' flank	4619	3419	95	9
Δ snm4 (<i>Sm3877</i>)	AflII, SphI	855-bp 5' flank	4619	2851	498	22
Δ Sm3882c (<i>snm7</i>)	AflII, NcoI	885-bp 3' flank	7552	1758	464	16
Δ mycP1 (<i>Sm3883c</i>)	PvuI	865-bp 5' flank	2294	1695	449	15

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Table 3. Constituents of 7H9 and Sauton's media and summary of experiments to uncover the precise component which regulates SmESAT-6 export.

Ingredients in 7H9 media (per liter)	Ingredients in Sauton's media (per liter)	REMOVED FROM 7H9 (<i>Sm ESAT-6 export</i> <i>still suppressed</i>)	ADDED TO SAUTON'S (<i>SmESAT-6 export</i> <i>still observed</i>)
5 ml glycerol	48 ml glycerol	*	*
0.1 g sodium citrate	2 g sodium citrate		
1 g potassium phosphate (monobasic)	0.5 g potassium phosphate (dibasic)		√
0.04 g ferric ammonium citrate	0.05 g ferric ammonium citrate		
0.05 g magnesium sulfate	0.5 g magnesium sulfate		
0.5 g glutamic acid	4g asparagine		
2.5 g sodium phosphate			√
5 g dextrose		√	√
5 g ammonium sulfate		√	√
0.001 g copper sulfate		√ **	√ **
0.001 g zinc sulfate		√ **	√ **
0.001 g pyridoxine		√ **	√ **
0.005 g calcium chloride		√ **	√ **
0.0005 g biotin		√	

* Using 48ml of glycerol in 7H9 media or 5ml of glycerol in Sauton's media had no effect on SmESAT-6 export.

** These four metal and vitamin supplements were added and removed individually and in concert, with no effect on SmESAT-6 secretion.

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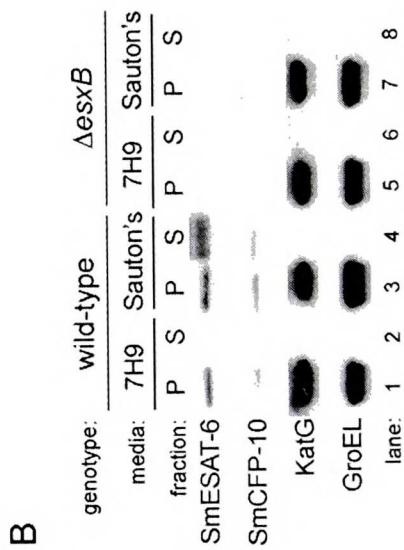
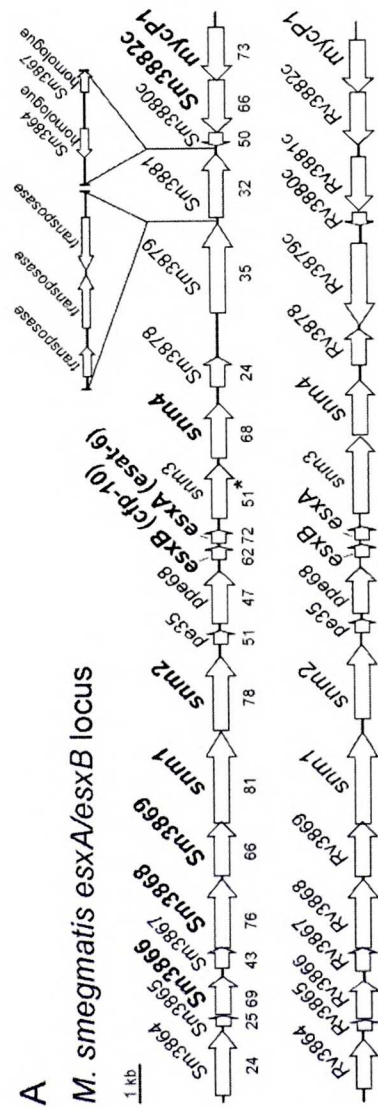
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Figure 1. *M. smegmatis* possesses a highly homologous *esxA/esxB* genomic locus and secretes SmESAT-6 and SmCFP-10 in a media-dependent manner.

(A) *M. smegmatis* genomic sequence (<http://www.tigr.org/tdb/ufmg/>) was obtained from TIGR, and *M. tuberculosis* genomic sequence was obtained from Tuberculist (<http://genolist.pasteur.fr/TubercuList/>). The numbers below each ORF from *M. smegmatis* denotes the encoded protein's percent identity (using the lalign program: http://www.ch.embnet.org/software/LALIGN_form.html) to its homologue in *M. tuberculosis* and the bolded gene names represent those included in this study. The asterisks indicate that while the *snm3* homologue from *M. smegmatis* is 67% identical to its counterpart in *M.tb*, it lacks the 577 bp N-terminal portion found in the *M.tb* gene. **(B)** Wild-type or $\Delta esxB$ cells were grown to late-log phase in 7H9 or Sauton's media, fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blot for the indicated proteins.





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Figure 1. Schematic diagram of the experimental setup.

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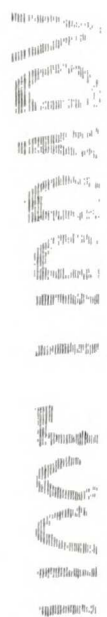
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Figure 2. In-frame deletion strategy and Southern blot analysis of mutants is shown.

(A) The in-frame deletion strategy involved PCR amplifying 800 bp 5' and 3' flanks to the wild-type gene of interest. The oligos were appropriately engineered to introduce a unique *Afl*III restriction digest site to ensure that the ligated 5' and 3' flanks would express an in-frame truncated gene product. Recombination between the genome and either 800 bp flank produced a kanamycin-resistant merodiploid strain, and counter-selection on sucrose for recombinants selected for either the desired in-frame deletion or the restored wild-type gene product. **(B)** Southern blot analysis of each deletion strain revealed the expected bands. Genomic DNA was digested with *Afl*III and one additional enzyme to confirm that the suicide plasmid had recombined in the proper location and that the *Afl*III site, thus likely the correct reading frame, were intact. For the *mycP1* Southern, genomic DNA was digested with *Pvu*I alone. For more detail, see Table 2.



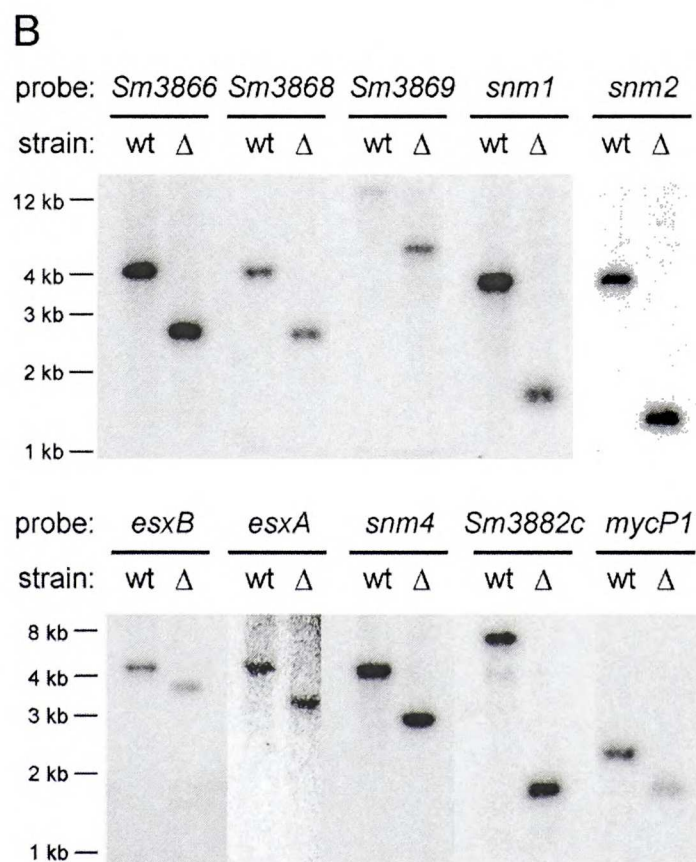
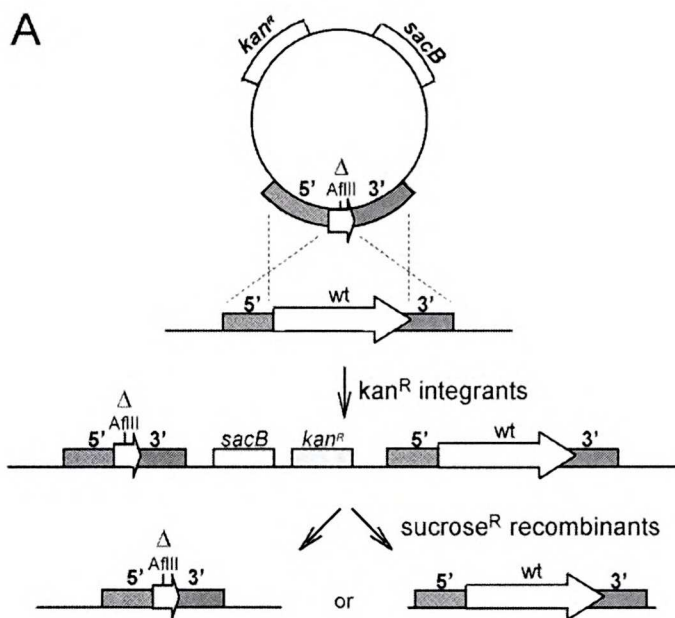


Figure 3. *M. smegmatis* secretes SmESAT-6 and SmCFP-10 in an Snm-dependent manner, and four additional uncharacterized genes are also required for SmESAT-6 and SmCFP-10 secretion.

(A) and **(B)** Wild-type and various mutant cells were grown in to late-log phase in Sauton's media, fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blot for the indicated proteins or by silver stain (right panel of **A**).

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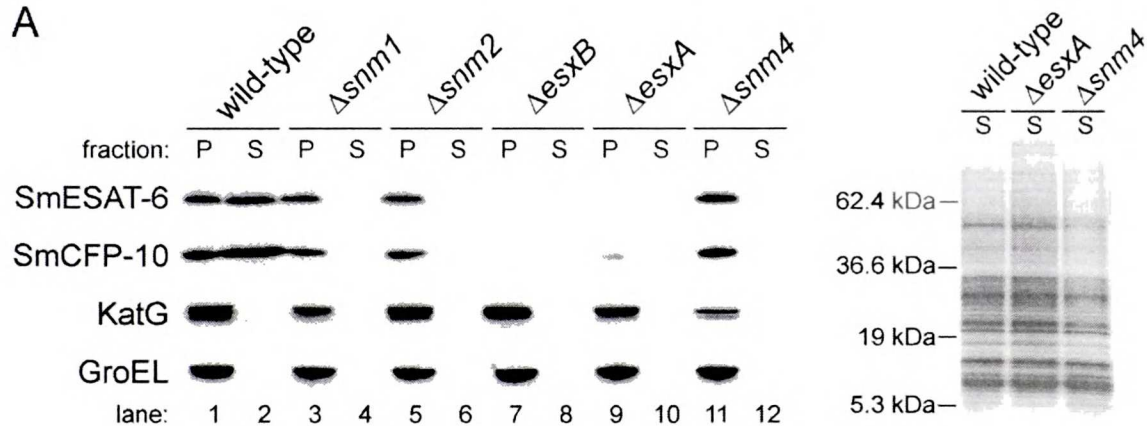
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Abstract

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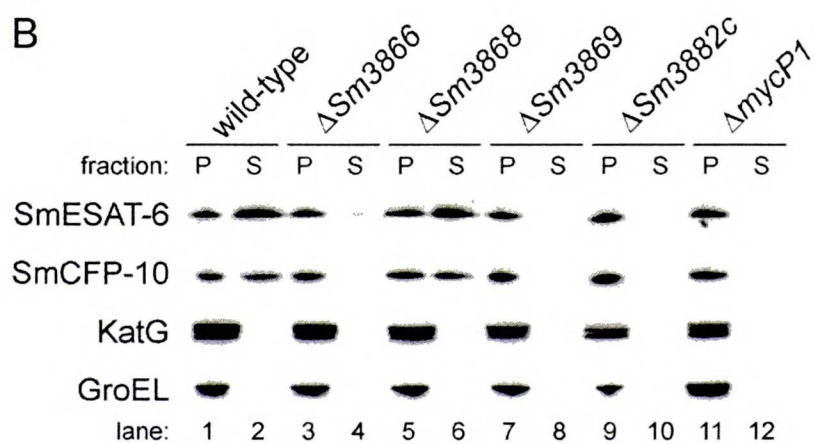


Figure 4. Complementation of mutant strains with individual genes confirms that the Snm secretion pathway functions in *M. smegmatis*.

(A & B) Various deletion strains were transformed with either an empty vector (pMJ13) or a multi-copy episomal complementation plasmid constitutively expressing each individual gene C-terminally tagged with myc. In the case of *Sm3882c* and *mycP1* complementation, the complementation plasmid expressed the individual genes tagged with 2x HA from the presumed endogenous promoter (505 bp fragment upstream of *mycP1*). **(C)** In order to test for *M. tuberculosis* (*M.tb*) ESAT-6 and CFP-10 secretion from *M. smegmatis*, cells were transformed with pMH406, which complements the *M. tuberculosis* *esxA/B* deletion (Guinn et al., 2004, data not shown). All cells were cultured in Sauton's media with kanamycin, fractionated, and analyzed by Western blot.



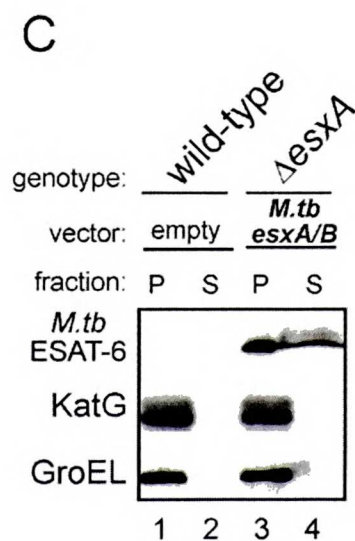
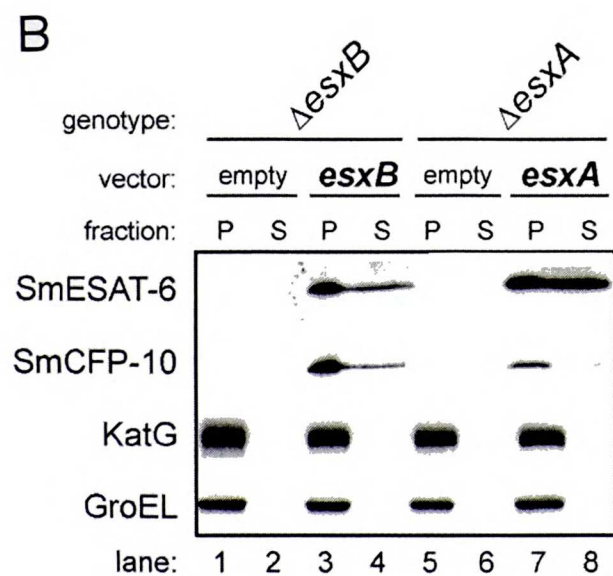
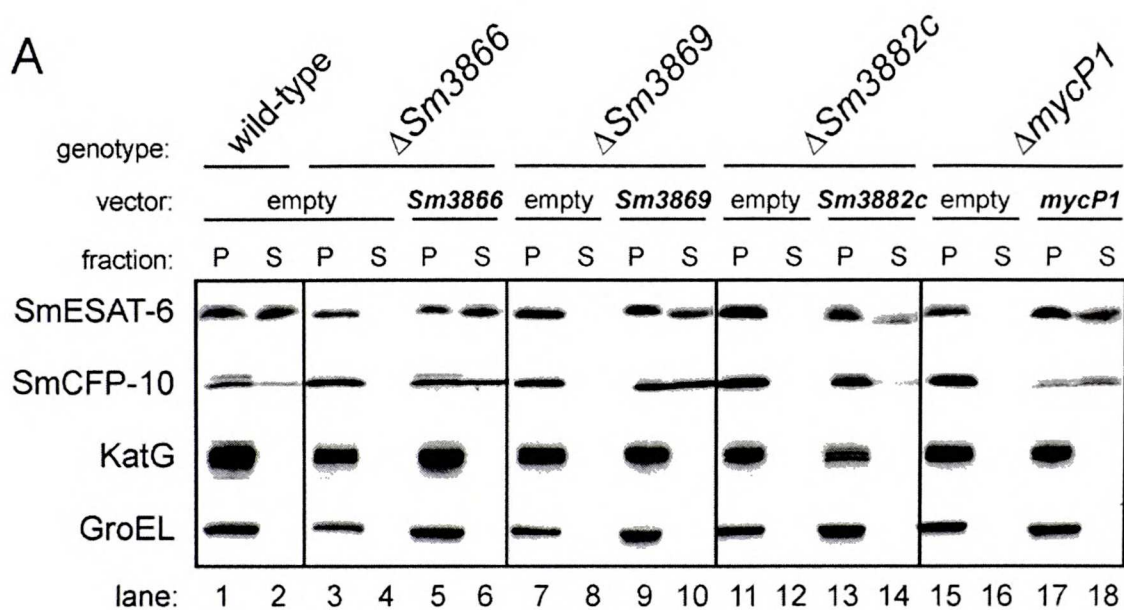




Figure 5. Model of the Snm secretion pathway in *M. smegmatis*.

See *Discussion* for details of the model. CM, cytoplasmic membrane; PG, peptidoglycan layer; mAG, mycolyl arabinogalactan layer. Transmembrane domains were predicted by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), and topology is inferred based on predicted function and some experimental evidence (Dave et al., 2002).



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35-44	30%
45-54	25%
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65-74	10%
75-84	5%
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CONCLUSIONS

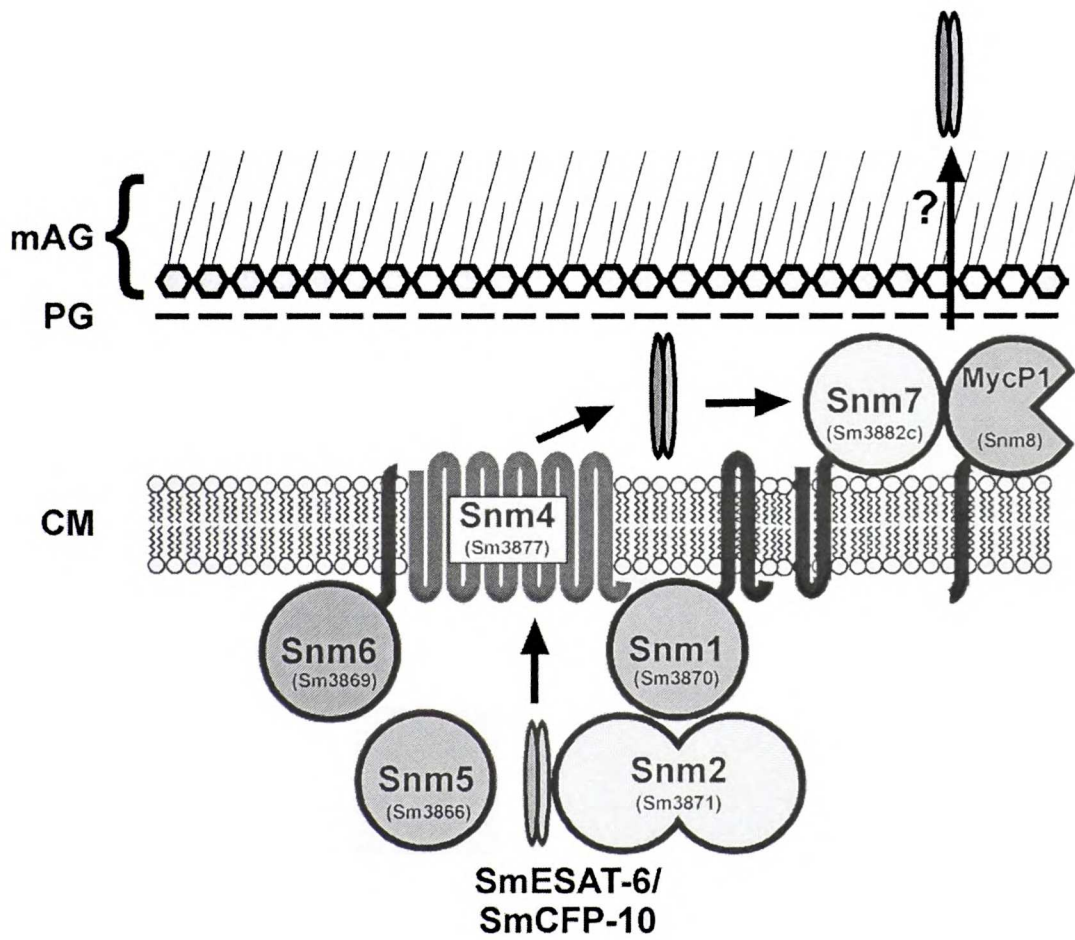
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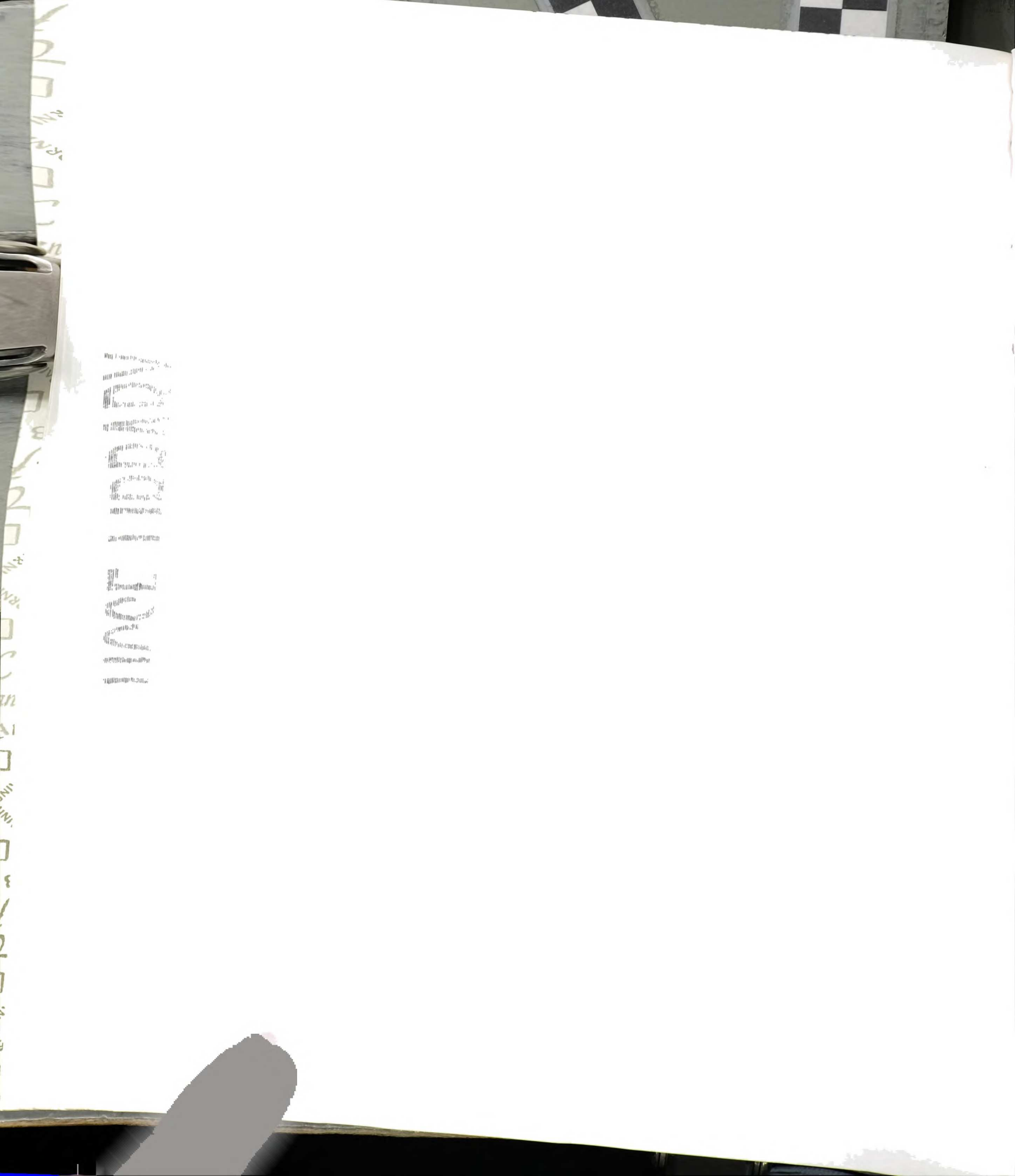


Figure 6. Timecourse of SmESAT-6 secretion from wild-type *M. smegmatis*.

At T=0, wild-type *M. smegmatis* was inoculated at a low O.D.₆₀₀ (0.05) into 200ml of Sauton's media. At the indicated time points, 50ml aliquots were collected, and whole-cell and STCF fraction were purified and analyzed as described in previously.

Figure 6. Timecourse of SmESAT-6 secretion from wild-type *M. smegmatis*.

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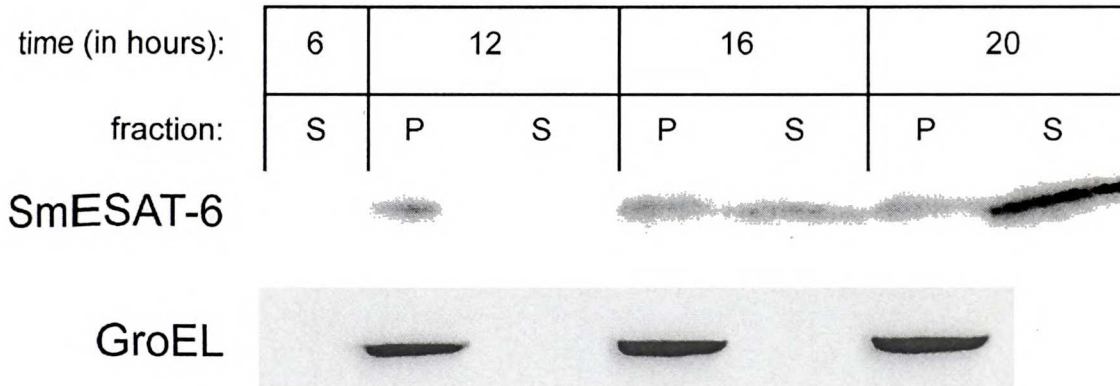
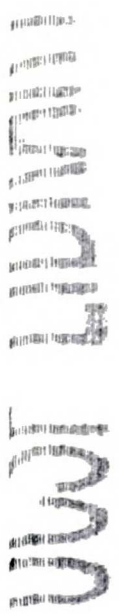
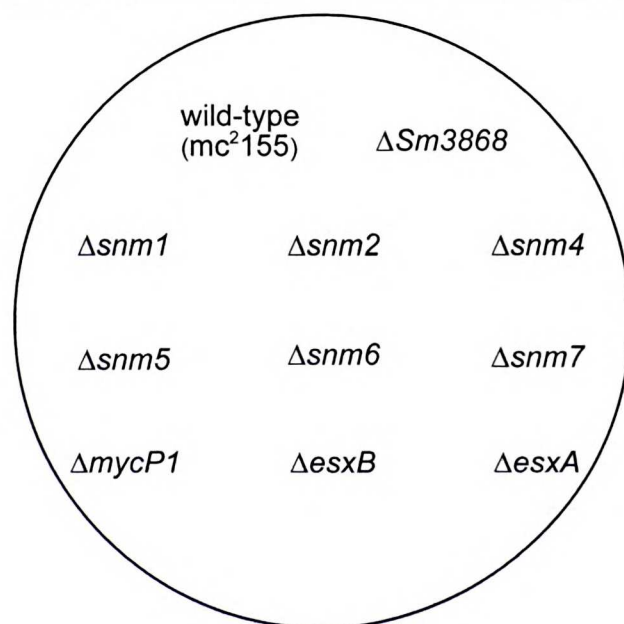
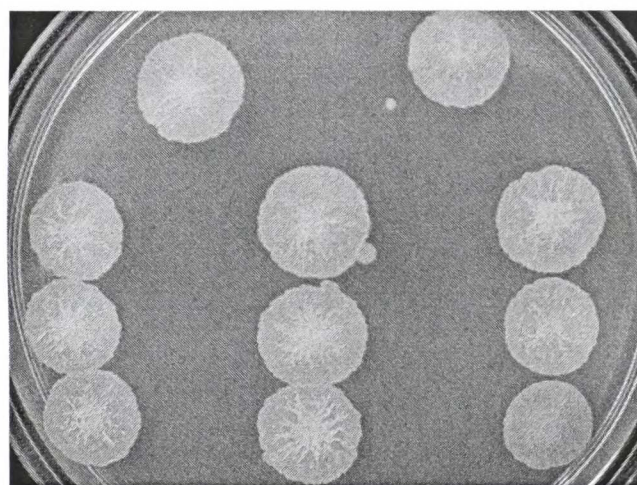
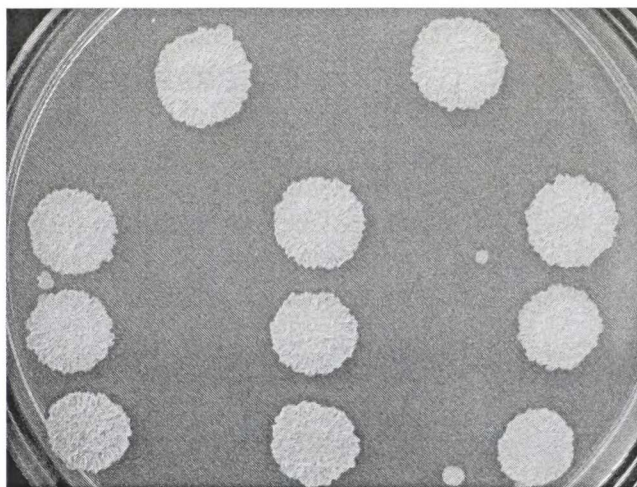




Figure 7. Colony morphology of *snm/esx* mutants.

Cells were spotted onto 7H10 plates (top panel) and Sauton's plates (middle panel) and grown for 3 days at 37°C. Tween-80 was not included in either plate, in order to more clearly discern the rough colony morphology of each strain. The lower panel is the key for the strains on each plate.





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Figure 8. Media mixing experiments reveal that 7H9 media exerts a dominant-negative effect on the Snm pathway.

Cells were first split into 3 cultures: 1) 7H9 alone, 2) Sauton's alone, or 3) equal parts (1x) 7H9 and Sauton's media. Cells grown in the mixed media doubled at approximately the same rate as those grown in 7H9 media alone (doubling rates of 2.9, 3.7, and 3.0 hours for 7H9, Sauton's, and mixed media, respectively), and harvest times were adjusted to insure that all samples had equivalent cell numbers. Whole-cell and STCF fractions were harvested and analyzed as described previously.





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Figure 1. Schematic diagram of the experimental setup. The subject is seated in a chair, viewing a video screen. The screen displays a target (a red dot) and a starting point (a green dot). The subject's hand is positioned at the starting point. The distance between the starting point and the target is 10 cm. The subject is instructed to move their hand from the starting point to the target. The video screen is 100 cm high and 100 cm wide. The starting point is 50 cm from the bottom edge of the screen. The target is 50 cm from the top edge of the screen. The subject's hand is 50 cm from the bottom edge of the screen. The distance between the starting point and the target is 10 cm. The subject is instructed to move their hand from the starting point to the target.

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Chapter 4

Subcellular Fractionation and Electron Microscopic Analysis Reveals New Characteristics of Snm Protein Secretion Pathway in *Mycobacterium* *smegmatis*

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Abstract

The mycobacterial cell wall serves an elaborate defensive barrier, yet these organisms have evolved an extensive secretory protein machine, which we have termed the Snm pathway, to deliver an ever-increasing list of protein effectors through the membrane and cell wall and into the host cell to mediate critical host-pathogen interaction *in vivo*. We have developed *Mycobacterium smegmatis* as a model system to understand this pathway, and our panel of *snm/esx* mutants with tagged complementing Snm constructs presented a unique opportunity to delineate how SmESAT-6 is transported across these outer cell layers and how the Snm machine assembles to mediate this process. We developed two novel fractionation procedures to localize SmESAT-6 and the Snm machine into five distinct fractions: cytosol, plasma membrane, cell wall, surface-exposed cell wall proteins, and the secreted culture filtrate fraction. This analysis demonstrated that SmESAT-6 is not enriched in either the membrane or cell wall fractions of wild-type or *snm/esx* mutant cells. Interestingly, components of the Snm machine did localize to distinct compartments of the mycobacterial cell. Additionally, we isolated several surface-exposed cell wall proteins whose localization required an intact Snm machine. Finally, our analysis of *snm* mutants by scanning electron microscopy led us to the inadvertent discovery of dramatic *snm*-independent extracellular filaments or fimbriae (EFs) and outer membrane vesicles (OMVs) on the surface of *M. smegmatis*.

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Introduction

The Snm alternative protein secretion pathway is a critical component of *Mycobacterium tuberculosis* virulence and host immune modulation (Stanley et al., 2003). Certain members of the Snm pathway (particularly Snm1/2 and ESAT-6 and CFP-10) are conserved in a wide range of medically relevant Gram-positive bacteria (*Mycobacterium* spp., *Clostridium* spp., *Corynebacterium diphtheriae*, *Bacillus anthracis*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Pallen, 2002). Additionally, *Legionella pneumophila*, a Gram-negative intraphagosomal pathogen, uses Snm1/2 and ESAT-6 homologues to mediate macrophage killing (Segal and Shuman, 1997). Recent work studying non-mycobacterial Snm-like pathways has reached different conclusions as to their direct link to pathogenesis. In *S. aureus*, the homologous Snm pathway is absolutely required for virulence, while partial ESAT-6 deletions in *L. monocytogenes* display unaltered pathogenesis (Burts et al., 2005; Way and Wilson, 2005). However, the abundance of homologues in numerous bacterial pathogens warrants further studies to define both the similarities and differences between the function of the Snm pathway during pathogenesis in these organisms. Furthermore, understanding the mechanism and function of the Snm pathway in these species will aid in the discovery of important new drug targets.

While the components involved in exporting ESAT-6 and CFP-10 and the other proposed Snm substrates, EspA and PE35 (Fortune et al., 2005), are only beginning to be identified, the underlying function of the Snm pathway during

Will the United States be able to meet the needs of the world's population in the future? The answer is yes, if we can find a way to produce more food and other goods without polluting the environment. The United States has a long history of producing food and other goods, but we have also been polluting the environment. We need to find a way to produce more food and other goods without polluting the environment. We need to find a way to produce more food and other goods without polluting the environment.

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Figure 1. Schematic diagram of the experimental setup for the study of the effect of the initial concentration of the monomer on the polymerization rate.

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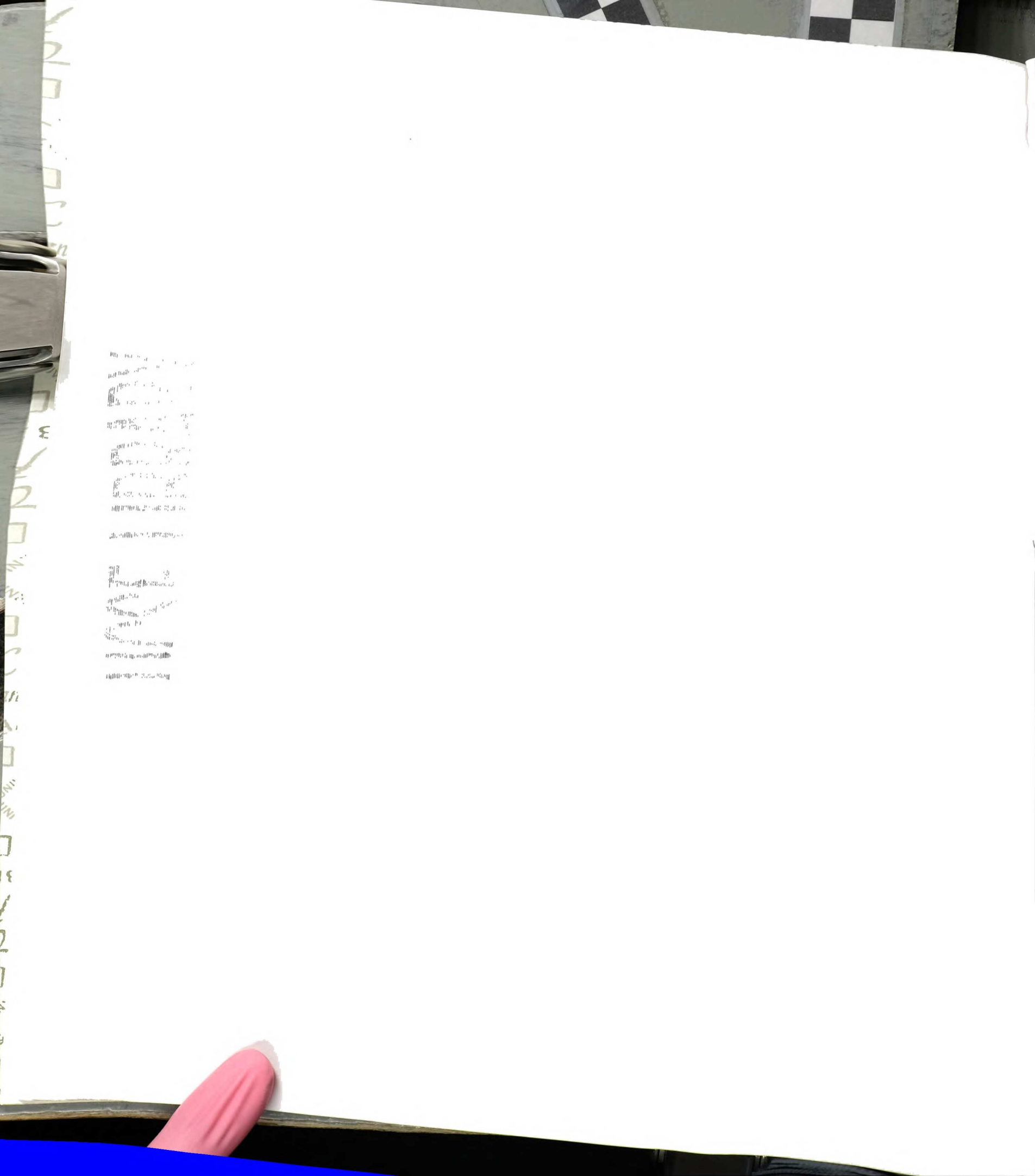
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virulence is hotly debated. Several proposed *in vivo* functions have been assigned to the most well-characterized *Sn*m pathway secretion substrate, ESAT-6. Its original isolation as a potent T-cell activator and immunodominant antigen pointed to a potential immunomodulatory role (Skjot et al., 2000; Sorensen et al., 1995). Several groups hypothesized that ESAT-6 itself may have hemolytic activity on host cells (Gao et al., 2004; Hsu et al., 2003). However, direct evidence for hemolytic activity is insufficient and the recently solved crystal structure of the ESAT-6 and CFP-10 dimer reveals no obvious membrane binding domains (Renshaw et al., 2004). Interestingly, Renshaw et al demonstrated cell-type specific surface binding of ESAT-6 and CFP-10 on macrophages, suggesting that they may interact with a host cell receptor to alter host signaling. However, the identity of this predicted receptor is unknown.

Additionally, ESAT-6 and CFP-10 may perform important functions to the bacteria themselves. Flint et al. recently reported that *M. smegmatis snm* mutants display increased conjugation efficiency and present evidence that *Sn*m secretion suppresses conjugation *in trans* (Flint et al., 2004). While they present no direct evidence, they hypothesize that ESAT-6 and CFP-10 may act as quorum sensing molecules in order to allow bacterial communication.

Alternatively, subcellular fractionation of *M. tuberculosis* reveals abundant levels of ESAT-6 in the cell wall, and certain *snm* mutants display altered colony morphology, prompting the speculation that ESAT-6 and CFP-10 secretion is merely a sloughing off of cell wall ESAT-6 and CFP-10 (Pym et al., 2002). However, recent data from this same group and others confounds this



hypothesis, as ESAT-6 and CFP-10 did not localize to cell wall in wild-type cells and only accumulated in the cell wall upon mutation or epitope-tagging (Brodin et al., 2005; VanderVen et al., 2005).

A related hypothesis, which may explain the ESAT-6 and CFP-10 cell wall localization, is that ESAT-6 and CFP-10, rather than secretion substrates, may function as part of the Snm secretory apparatus at the surface of the mycobacteria. Diverse cell surface structures, such as pili, needles, or filaments, are important components of Type II, Type III, and Type IV alternative secretion systems in Gram-negative bacteria (Christie, 2004; Ghosh, 2004; Peabody et al., 2003; Remaut and Waksman, 2004). In many cases, these structures serve as continuous conduits for secretion substrates and/or mediate important host or bacterial cell contacts. Interestingly, mycobacterial extracellular filaments have been identified using scanning electron microscopy, but the composition or function of these structures has not been determined (Merkal et al., 1973). Whether these structures participate in the mycobacterial Snm secretion pathway is unclear.

As we have validated *M. smegmatis* as a useful model system to understand the Snm pathway, we sought to take advantage of our numerous *snm* mutants to determine 1) if they display altered gross morphology, particularly at the cell surface, using scanning electron microscopy and 2) if SmESAT-6 is a *bona fide* cell wall protein in wild-type *M. smegmatis* or perhaps trapped in the cell wall in any *snm* mutants. Using our improved fractionation protocols, we also localized several Snm secretory proteins to distinct locations

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in the cell and uncovered several *Snm*-dependent surface-localized protein species. These studies contribute to our working model of the *Snm* pathway and suggest that the *Snm* pathway must be tightly regulated to coordinate *SmESAT-6* and *SmCFP-10* export across the entire cell wall into the extracellular milieu.

Results

Scanning electron microscopy of *M. smegmatis* wild-type isolates and *snm* mutants.

In order to investigate whether any extracellular structures are present in our wild-type *Mycobacterium smegmatis*, we performed scanning electron microscopy (SEM) using carbon/platinum coating which is well suited for preserving and visualizing extracellular structures (Gebhart et al., 1979). As our wild-type *M. smegmatis* mc²155 is a laboratory strain selected for its efficient transformation phenotype, we also characterized the parental strain, mc²1 or ATCC607, and ATCC19420, a virulent clinical isolate in goldfish and felines (Talaat et al., 1999; Alander-Damsten et al., 2003).

As shown in Figure 1, SEM analysis of wild-type mc²155 reveals interesting features at the mycobacterial cell surface. First, we discovered that dramatic extracellular filaments or fimbriae (EFs) protrude from the cell wall of a subset of wild-type cells. As shown in Figure 1A and B, these EFs are quite wide, measuring approximately 60-90 nm in diameter, and display variable

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length, reaching as far as several microns in length (panel A). These findings are consistent with a hollow bundled filament previously described in *M. paratuberculosis* (Merkal et al., 1973). Interestingly, these EFs mediate both cell-to-cell and cell-to-substrate contacts (as indicated in the Figure 1 legend), which might suggest a role in motility, cell-to-cell communication, or group behavior.

In addition to EFs, we observed extensive membrane “blebbing” at the surface of the mycobacteria (Figure 1A, B, and D). These structures are readily apparent on the surface of most cells and might contribute to the high degree of extracellular debris present in the samples (Figure 1A and B). As the mycobacterial cell wall consists largely of lipid material, and there are reports of lipid secretion beyond the cell wall *in vitro* and *in vivo* (Cox et al., 1999; Beatty et al., 2000), we hypothesize that these structures are outer-membrane vesicles (OMVs). In some cases, these OMVs co-localize with EFs (see Figure 1B, 2B, 3B, 5A&B), which might indicate coordinated function.

SEM analysis of the mc²155 parental strain, mc²1, revealed significant phenotypic differences between the strains. As shown in Figure 2A & B, this strain exhibits dramatic increases in both the number and length of the EFs, many of which are extensively branched. Furthermore, the magnified image in Figure 2B indicates that the mc²1 cells possess a more rugose cell surface than its relative, mc²155. These findings indicate that during the creation of the laboratory mc²155 strain, partial loss of EFs and dramatic cell wall alterations likely occurred, which might explain why mc²155 is highly transformable.

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SEM analysis of the *M. smegmatis* clinical isolate ATCC19420 revealed an immense number of EFs (Figure 3). As opposed to mc²155 or mc²1, virtually every cell possessed multiple branched filaments, which produced a meshwork of filaments surrounding the colony of cells (panel D). The ATCC19420 cells are less rugose than mc²1, but OMVs and extracellular debris are readily apparent in this strain. Unfortunately, both mc²1 and ATCC19420 were refractory to transformation and phage-mediated DNA delivery, which prevented further genetic work to discern the components and regulators of these EFs and OMVs.

As extracellular filaments or pili are often linked with alternative protein secretion pathways, we next sought to determine if the *Snm* pathway might regulate or utilize these EFs during secretion. As the clinical isolates display significantly more EFs by SEM, we analyzed their cell pellets and secreted protein fractions for the presence of SmESAT-6. Interestingly, both mc²1 and ATCC19420 were unable to secrete SmESAT-6 into the extracellular milieu (Figure 4). After normalizing based on the *KatG* cytoplasmic loading control, these isolates also synthesize significantly lower intracellular levels of SmESAT-6, which might also contribute to the apparent secretion defect. These results clearly demonstrate that SmESAT-6 is not a constituent of the EFs; rather, it might indicate that SmESAT-6 secretion is a negative regulator of EF biogenesis.

In order to determine if SmESAT-6 secretion negatively regulates EF formation, we performed SEM on our panel of *snm* and *esx* mutants. Several

representative images are shown in Figure 5, but no discernible phenotypes emerged from this analysis. The appearance of EFs and OMVs are clearly not abolished by the loss of *Snm* function. Conversely, the *snm* or *esx* mutants did not possess discernibly more EFs at their cell wall surface. Therefore, while the loss of SmESAT-6 secretion correlates with the clinical isolate's ability to elaborate extensive EFs, loss of SmESAT-6 alone does not cause hyper-filamentation.

However, several *snm* mutants did display novel phenotypes by SEM. Analysis of the $\Delta snm5$ strain revealed abundant "mini-cells," which might indicate a septation defect (Figure 5E). However, these "mini-cells" were not present in all $\Delta snm5$ samples, suggesting that this may represent only a small proportion of the population. Another interesting morphology, depicted in panel F, is a dramatically swollen $\Delta esxB$ cell. Interestingly, exogenous expression of the *M. tuberculosis* Ser/Thr kinase PknF in *M. smegmatis* also induced cell swelling (Deol et al., 2005). However, like the "mini-cells," the swollen morphology was not present in all samples, so we can not directly link loss of *esxB* to cell swelling or rule out that a wild-type cell can similarly swell.

Using this approach we were unable to demonstrate reproducible differences in the mycobacterial cell surfaces in our *snm/esx* mutants. Our findings using SEM, combined with our colony morphology studies (Chapter 3, Figure 7) indicate that our *snm* mutant strains are not profoundly altered in their cell morphology. However, our SEM analysis did identify striking cell wall structures not previously described in *M. smegmatis*. Our discovery of these

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EFs and OMVs at the cell surface also revealed extensive phenotypic differences between wild-type strains.

Analysis of SmESAT-6 localization by subcellular fractionation.

As ESAT-6 is a proposed cell wall protein in *M. tuberculosis* (Pym et al., 2002), we sought to elucidate the localization of SmESAT-6 within wild-type *M. smegmatis* cells using an established differential centrifugation protocol. Briefly, cells are disrupted by probe sonication, unbroken cells are removed by a low-speed spin, then cell wall and membrane fractions are pelleted by two sequential ultra-centrifugation steps. Unfortunately, cell wall fractions purified in this manner were significantly contaminated with KatG, a strictly cytosolic protein in *M. smegmatis* (data not shown, Raynaud et al., 1998).

As unbroken cells co-sedimenting with the cell wall fraction are likely responsible for the KatG contamination, we chose instead to use bead beating as our method of cell disruption. Additionally, we lowered the total number of cells ten-fold, added four additional low-speed spins to ensure no unbroken cells remained in the lysate fraction, and rigorously washed the cell wall and membrane fractions. In order to determine enrichment of cell wall proteins, we utilized antibodies to the major cell wall porin, MspA. This well-studied porin resides on the surface of the bacteria buried into the cell wall, where it imports small hydrophilic molecules (Faller et al., 2004; Stahl et al., 2001). In order to judge any membrane contamination in the cell wall fractions, we fractionated cells expressing the exogenous *M. tuberculosis* MmpL7 lipid transporter. This

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2. *Definition 2.1* (Hilbert, 1902): Let $\mathcal{H}$ be a Hilbert space. A sequence $\{e_n\}_{n=1}^\infty$ in $\mathcal{H}$ is called an orthonormal basis (ONB) if it satisfies the following conditions:

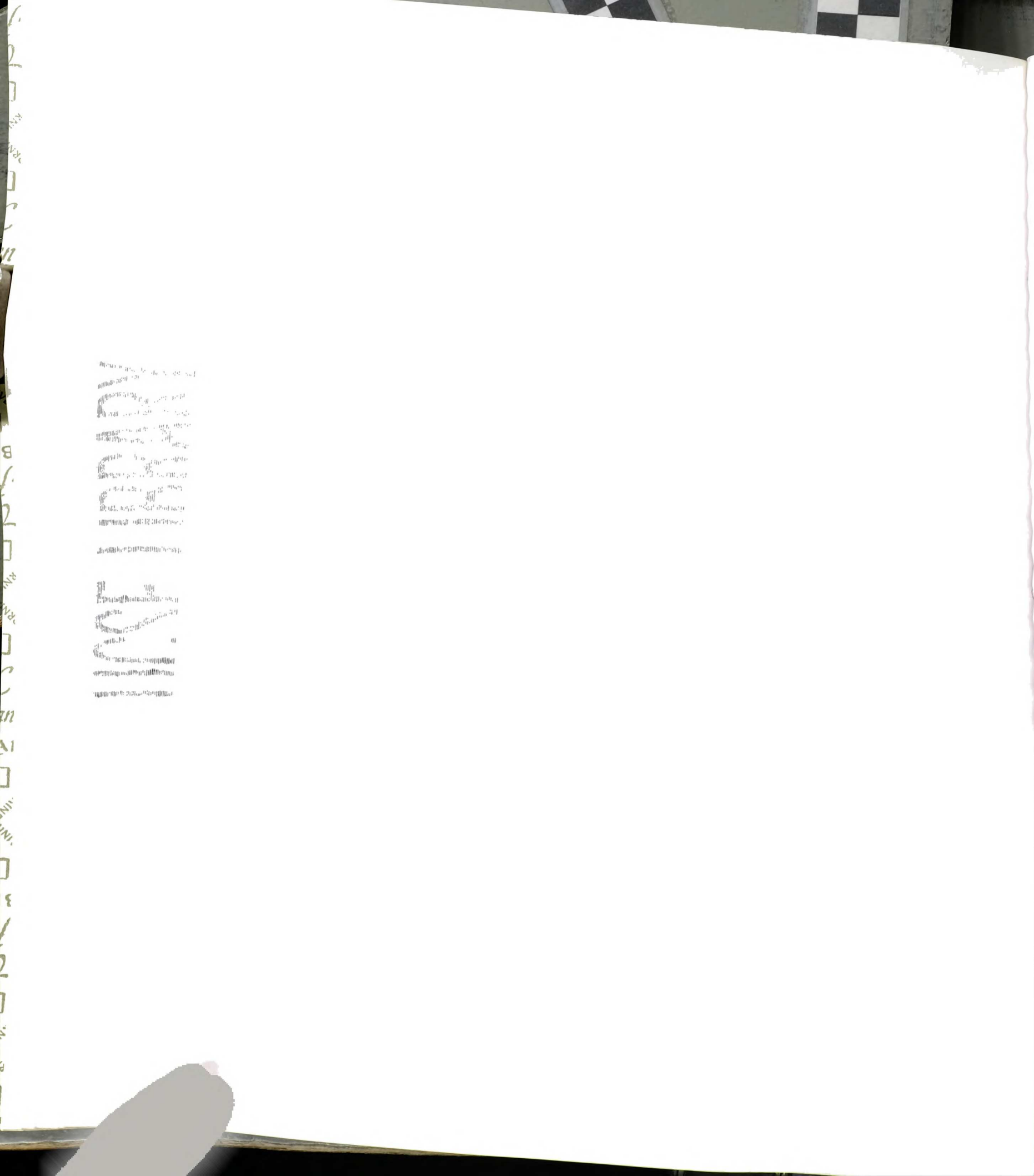
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well-characterized protein contains 12 transmembrane domains and contains both periplasmic and cytosolic soluble domains.

Using our improved protocol, purified cell wall fractions, normalized for protein content and analyzed by Western blot, contain low levels of KatG and enrichment of MspA when compared to the cytosolic or membrane fractions (Figure 6A). Furthermore, silver staining of each fraction by SDS-PAGE revealed distinct protein profiles (data not shown) which suggests that our improved fractionation protocol was indeed isolating distinct cellular compartments.

Interestingly, MspA, which functions as a porin in the cell wall outer-membrane, is also a major component of the secreted short-term culture filtrate (STCF) fraction (Figure 6A). We were at first perplexed by this finding, since this might indicate that true cell wall proteins may contaminate our STCF fractions. However, in light of our SEM results, this is additional evidence that cell wall material is released into the extracellular milieu, and MspA at the cell wall surface may be packaged into OMVs during their release into the STCF fraction.

We wanted to determine if MspA “secretion” is an active process and rule out trivial explanations, so we cultured wild-type cells in either Sauton’s or 7H9 media and analyzed the secreted proteins (Figure 6B). Surprisingly, growth in 7H9 media caused significantly less MspA release when compared to cells cultured in Sauton’s media. This indicates that shaking during culturing or use of the Tween-80 detergent during early culturing steps is not responsible for



MspA release. Furthermore, equivalent levels of MspA were recovered from the cell walls of wild-type cells grown in either media (data not shown); therefore, the media-dependent MspA release is not a result of altered total MspA levels with the cell wall. It is noteworthy that the same culturing conditions that suppress SmESAT-6 secretion also prevent MspA release, which might indicate that the pathways are linked or that a global regulator suppresses both pathways when cultured in 7H9 media.

In order to judge both our ability to purify membrane proteins and the extent of membrane contamination in our cell wall fractions, we fractionated cells expressing a marker for the membrane fraction, MmpL7-HA. While MmpL7-HA is absent from the cytosolic fraction, it predominately localized to the cell wall fraction (Figure 6C). Over-exposure of the Western blot did reveal low levels of MmpL7-HA in the membrane fraction (Figure 6C, lower panel), but these results suggest that membrane proteins co-sediment with our cell wall fractions, which are nevertheless enriched for MspA and devoid of KatG,

In order to determine the localization of SmESAT-6 in wild-type *M. smegmatis*, these same fractions were probed for SmESAT-6, revealing exclusive localization to the cytosolic and STCF fractions (Figure 6A). Cell wall samples concentrated ten-fold and analyzed by Western blot for SmESAT-6 were still negative (data not shown) which suggests that SmESAT-6 is not a *bona fide* cell wall protein in wild-type *M. smegmatis*. Furthermore, SmESAT-6 is absent from both the plasma membrane fraction and any of its contaminants in the cell wall fraction.

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Subcellular localization of SmESAT-6 in *snm* mutants.

Using cell fractionation, we have demonstrated that SmESAT-6 is not a major component of the wild-type cell wall, but we were curious if any of our panel of *snm* mutants may trap intermediate transport steps in the Snm pathway. Therefore, we fractionated our panel of *snm* and *esx* mutants and probed each fraction for SmESAT-6, MspA, and KatG. As shown in Figure 7, cell wall fractions from each mutant were negative for SmESAT-6 and possessed similar levels of the control proteins, KatG and MspA. Importantly, SmESAT-6 is a highly abundant component of the cytosol in each *snm* mutant and absent in *esx* mutants (see Chapter 3, Figure 3). Although a negative result, this data is consistent with the hypothesis that the Snm secretory apparatus transfers ESAT-6 and CFP-10 directly from the cytosol into the extracellular milieu. However, we cannot exclude the possibility that additional unknown *snm* mutants may indeed trap transport intermediates of SmESAT-6 within the cell wall.

Similar to the Snm pathway, MspA secretion is largely suppressed in 7H9 growth media; therefore, we hypothesized that SmESAT-6 secretion may be required for MspA release. However, the *snm* mutants released similar levels of MspA when compared to wild-type cells, suggesting that the loss of SmESAT-6 secretion alone does not alter MspA release (data not shown). This finding matches our SEM phenotypic analysis of the *snm/esx* mutants, which display no alteration in OMV biogenesis. However, the media-dependence and

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snm-independence of MspA release raises the intriguing possibility that SmESAT-6 may be packaged into OMVs, whose formation and secretion are Snm-independent.

Subcellular localization of the Snm secretory apparatus.

Using our improved fractionation procedure, we next sought to determine the localization of the individual Snm machinery components in the mycobacterial cell. In order to mimic endogenous expression levels with our epitope-tagged Snm proteins (from Chapter 3, Figure 4), we created strains with integrated plasmids expressing tagged Snm5, 7, and mycosin-1 under the control of each gene's endogenous promoter. These constructs complemented the SmESAT-6 secretion defect in their appropriate knock-out strains (data not shown). Furthermore, these constructs expressed detectable levels of each Snm protein, and their measured mass, judged by SDS-PAGE migration, closely matched their predicted sizes (measured mass vs. predicted mass of 35.1 vs. 33.8kD for Snm5; 54.3 vs. 54.7kD for Snm7; and 54.7 vs. 50 kD for mycosin-1).

Fractionation and Western blot analysis revealed differential localization of the Snm machinery. As shown in Figure 8, myc-tagged Snm5 is exclusively cytosolic, while HA-tagged Snm7 and mycosin-1 localize to the cell wall fraction. As our cell wall fractions contain membrane contaminants, we cannot discern if Snm7 and mycosin-1 truly reside in the cell wall or plasma membrane, though low levels of Snm7 are apparent in the plasma membrane fraction.

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However, these results do suggest that they may function coordinately and distinctly from *Snm5* and other parts of the *Snm* machine.

Analysis of cell wall surface-exposed proteins in *snm/esx* mutants.

While SmESAT-6 is not a major component of the cell wall, as determined by subcellular fractionation, we cannot exclude the possibility that SmESAT-6 is loosely associated with the cell wall or its surface upon or after secretion. This association may be sufficiently weak that the process of bead beating may disrupt its interaction. Accordingly, we sought to develop an alternate approach to probe the outermost layer of the cell wall for the presence of SmESAT-6. Using the same principle as our cell wall lipid extraction from Chapter 2, we developed a gentle extraction procedure to remove solvent-accessible cell wall proteins using detergent and mechanical disruption. Ultimately, pulsed vortexing of cells in PBS with 0.2% Tween-80 extracted significant and reproducible levels of MspA (Figure 9B).

In order to determine if SmESAT-6 was loosely associated to the cell surface, we probed the wild-type and $\Delta snm4$ surface-exposed cell wall fractions for SmESAT-6. Our initial results, with separation on 4-20% gradient SDS-PAGE gels, suggested that SmESAT-6 might be released from the cell wall surface in an *snm4*-dependent manner (Figure 9A). However, further analysis using 15% SDS-PAGE gels revealed that at least three proteins are present, and their sizes (11.5kD – 14.2 kD MW range) are inconsistent with reported sizes of ESAT-6 in other mycobacteria (Figure 9B, Okkels et al., 2004). As

Philosophy of Science
The philosophy of science is a branch of philosophy that studies the foundations, methods, and implications of science. It seeks to understand the nature of scientific knowledge and the relationship between science and other domains of human inquiry.

Science and Philosophy

The relationship between science and philosophy is a complex one. On the one hand, science provides empirical data and theories that can inform philosophical inquiry. On the other hand, philosophy provides conceptual frameworks and critical analysis that can guide scientific research.

these Westerns were probed with partially purified polyclonal SmESAT-6 antibodies (generated with Freund's adjuvant), these species may either represent novel modified forms of SmESAT-6 or other immunogenic cell wall proteins present in the adjuvant. Efforts to purify and identify these *snm*-dependent cell wall proteins are ongoing, but these findings might suggest that multiple *Snm* substrates exist in *M. smegmatis*. Their elucidation will assist in our understanding of the secretion mechanism and perhaps the underlying biological significance of the *Snm* pathway in this soil saprophyte.

Discussion

Our choice of *M. smegmatis* as a model organism allowed approaches which are technically difficult in pathogenic mycobacteria. Our SEM analysis revealed dramatic extracellular filaments or fimbriae (EFs) that have been ignored in the mycobacterial field since their initial description in 1973 (Merkal et al., 1973). These filaments, which are most visible in clinical *M. smegmatis* isolates, often reach several microns in length, and their diameter is consistent with bundled fimbriae. They are also present in our lab strain, mc²155, though at drastically lowered levels. This may indicate loss of function during the repeated passaging required to derive this strain in the laboratory.

What are the components that make up these bundled filaments? Our attempts to pellet or purify these structures were unsuccessful, and the genetic intractability of the clinical isolates precluded any genetic studies. However, several lines of evidence suggest that these structures are secreted by a

dedicated pathway, termed the tight-adherence or tad pathway (Kachlany et al., 2001). This pathway, originally described in the virulent Gram-negative *Actinobacillus actinomycetemcomitans*, secretes two related pilin-like proteins, Flp1 and Flp2, which assemble into bundled extracellular pili. These bundled pili and their associated secretion machine (TadA-G,V,Z) mediate biofilm formation and are strictly required for virulence in animal models of periodontal disease (Schreiner et al., 2003; Wang and Chen, 2005). The genomic locus encoding the Tad pathway is a pathogenicity island that has spread by horizontal transfer to numerous Gram-positive and Gram-negative human pathogens (Planet et al., 2003), and remarkably, both *M. tuberculosis* and *M. smegmatis* possess clear homologues of *flp* and certain *tad* genes (*tadA-C* and *tadZ*) (see Figure 10, Kachlany et al., 2001). While these genes are uncharacterized in mycobacteria, an intriguing possibility is that these bundled pili may function during tuberculosis pathogenesis.

An unexpected finding from these experiments was evidence for outer-membrane vesicles (OMVs) in mycobacteria. Using two independent approaches, we demonstrated significant release of cell wall material. SEM analysis revealed OMVs and extracellular debris in virtually all fields examined. Additionally, the outer cell wall porin, MspA, is released into the STCF fraction from its predicted cell wall surface location (Figure 6). This is unexpected, as this porin, which must function in the cell wall to import small solutes, is predicted to be largely buried in the outer-membrane of *M. smegmatis* based on X-ray crystallography (Faller et al., 2004). Our combined results may indicate

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that MspA is included in outer-membrane vesicles where it might function beyond the mycobacterial cell wall. Attempts are ongoing to purify MspA-associated structures, which might co-localize with the OMVs present in our SEM samples. Importantly, the secretion of outer-membrane vesicles has been implicated in the delivery of virulence factors in other Gram-negative pathogens such as enteropathogenic *E. coli*, *Helicobacter pylori*, and *Shigella flexneri* (Wai et al., 2003). Recent work from our lab and others has provided examples of lipid and protein secretion both during *in vitro* culturing and *in vivo* macrophage growth (Cox et al., 1999; Beatty et al., 2000), and EFs and OMVs on the *M. tuberculosis* cell surface have recently been discovered using SEM analysis (Dahl, 2005). Therefore, future work in *M. tuberculosis* should focus on determining if OMVs or EFs mediate the delivery of certain proteins or lipids and if they function during tuberculosis pathogenesis.

We approached these SEM experiments in order to understand more details of the *Snm* pathway in *M. smegmatis* but were unable to link SmESAT-6 secretion directly to EF biogenesis. SEM analysis of *snm/esx* mutants did not reveal dramatically increased levels of EFs. We envision two explanations for this: first, this may represent a shortcoming of the SEM technique, as variability in sample preparation and limited sampling size might obscure any subtle differences. Eliminating the potential for sample-to-sample variability was even more problematic in *mc²155* and its *snm* mutants, as only a small proportion of cells possessed EFs or unique phenotypes (see Figures 1 & 5). Alternatively, the *Snm* pathway may indeed be involved in some aspect of EF biogenesis. In

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our effort to characterize the mc²1 and ATCC19420 strains, we uncovered that they were incapable of SmESAT-6 secretion and synthesis. While this is merely correlative, it is noteworthy that the mc²155 parental strain (mc²1) either originally lacked SmESAT-6 secretion or lost the ability over subsequent lab passaging.

With respect to OMV secretion, no single *snm* mutant lacked membrane “blebbing” based on SEM analysis or were incapable of MspA sloughing based on Western blot analysis of their STCF fractions. Yet, culturing in 7H9 media, which suppresses the *Sn*m pathway, similarly prevented MspA sloughing, suggesting that its release might be regulated in a similar fashion. Furthermore, we cannot exclude that the *Sn*m pathway may, in fact, regulate EF or OMV pathways, as *M. smegmatis* possesses multiple *Sn*m pathways and *Esx* homologues which may function redundantly in these pathways. This might indicate that our ATCC strains and 7H9 culturing conditions globally inhibit the *Sn*m pathway, causing altered MspA secretion and EF structures. Ultimately, future work must purify both the EFs and OMVs and elucidate their constituents, both lipidic and proteinaceous.

Our use of *M. smegmatis* as a model system has also contributed more details to our working model of the *Sn*m pathway (Figure 11). As predicted from recent structural and biochemical work, SmESAT-6 is not an integral component of the mycobacterial cell wall or cell membrane. Our use of the appropriate fractionation controls gave us confidence in these findings, and ultimately allowed our discovery of the distinct compartmentalization of the *Sn*m

machine. Using fractionation analysis, Snm5 localizes to the cytosol, while Snm7 and mycosin-1 are predominantly cell wall proteins. As Snm5 is predominately cytoplasmic, we predict that it functions in early secretion events, such as targeting or ATP-generation, which is consistent with its homology to ABC transporters (Gey Van Pittius et al., 2001). Several lines of evidence suggest that Snm7 and mycosin-1 are, in fact, present in the cell membrane: First, both these proteins have predicted transmembrane domains, and in the case of mycosin-1, its C-terminal transmembrane domain must be present in our C-terminally tagged protein. Second, the exogenous MmpL7 lipid transporter also co-localized to the cell wall fraction, suggesting membrane contamination. As MmpL7, Snm7, and mycosin-1 all contain predicted periplasmic domains, we hypothesize that these domains may interact with cell wall proteins with sufficient strength to survive the fractionation procedure. In the case of Snm7 and mycosin-1, this tight contact beyond the cytoplasmic membrane may provide a continuous conduit for SmESAT-6 and SmCFP-10 through the mycobacterial cell wall. We cannot exclude the possibility that mycosin-1 auto-proteolysis or Snm7 proteolysis may release them from the membrane into the cell wall (Figure 11). Indeed, there is some evidence that mycosin-1 is present in the *M. tuberculosis* cell wall (Dave et al., 2002), but these investigators used a flawed fractionation protocol and included no localization controls. Ultimately, further purification strategies will identify the source of the cell wall contaminants and allow confident determination of the localization and assembly of the Snm membrane complex.

To further understand the *snm*-dependent localization of any surface-exposed cell wall proteins, we developed a novel extraction procedure to purify any detergent-extractable cell wall proteins. This procedure released known cell wall proteins and several *snm*-dependent surface-exposed cell wall proteins (Figure 11). The shortcomings of our polyclonal antibodies prevented us from distinguishing if these proteins are several related forms of the same protein (perhaps modified forms of SmESAT-6) or multiple independent protein species. Interestingly, glycosylation of a small proportion of ESAT-6 was recently described in *M. tuberculosis* (VanderVen et al., 2005); therefore, these surface-bound species may be glycosylated forms of SmESAT-6. However, multiple *Sn*m substrates exist in *M. tuberculosis* (Fortune et al., 2005; Macgurn, J.A. and Raghavan, S., personal communication), so these proteins might be conserved and secreted in *M. smegmatis*. Biochemical purification and mass spectrometric characterization will identify these species and determine how they are targeted and exported by the *Sn*m pathway in *M. smegmatis*.

Materials and Methods

Scanning electron microscopy analysis

Various *M. smegmatis* strains were grown for 3 days on 7H10 or Sauton's plates, with or without 0.05% Tween-80. Individual colonies were fixed with osmium tetroxide vapours, glutaraldehyde, rinsed with Na-Cacodylate buffer, dehydrated with ethanol/water rinses (35, 35, 70, 80, 90, and 100%

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ethanol), and critical point dried. The samples were coated by electron beam evaporation with 2-3 nm platinum, followed by 10 nm of carbon in a Balzers 301 freeze etch unit. High resolution imaging was done with Hitachi S5000 SEM using either a secondary electron or backscatter detector.

***M. smegmatis* culturing and subcellular fractionation**

M. smegmatis was inoculated from a single colony and grown to mid-log phase ($OD_{600}=0.6-0.8$) in 7H9 media supplemented with 0.05% Tween-80 (Fisher Scientific). Cells were washed and inoculated in Sauton's media supplemented with 0.05% Tween-80. Upon reaching mid-log phase ($OD_{600}=0.6-0.8$), cells were washed and inoculated in 50 ml Sauton's media without Tween-80 and grown to an $OD_{600}=0.8$. Cells were harvested by centrifugation and the cultured media supernatant was filtered through a $0.22\mu\text{m}$ filter and concentrated using an Amicon Ultra-15 (5,000 MWCO, Millipore). The cell pellet was washed once with PBS, then resuspended in 1ml of PBS buffer containing proteinase inhibitors (pepstatin, leupeptin, and PMSF), DNase, and RNase. After adding 200 μl of 100- μm zirconium/silicone beads, the cells were homogenized for 3x1-minute periods using a Mini-Beadbeater (Biospec Products). Unbroken cells were removed by 4 successive low-speed (3,000 x g) 10-minute spins. The resulting whole-cell lysate was then centrifuged at 35,000 x g for 1 hour to sediment the cell wall fraction. The cell wall fraction was washed once with PBS, re-pelleted, then resuspended in PBS containing proteinase inhibitors. The supernatant was then spun at 100,000 x g

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for 4 hours to sediment the membrane fraction, which was washed once with PBS, re-pelleted, then resuspended in 0.01M ammonium bicarbonate. The remaining supernatant represented the cytosolic fraction. All fractions were quantified using MicroBCA (Pierce) and, after protein normalization, loaded on 4-20% Tris-HCl SDS-PAGE Criterion gels (Biorad). For immunoblotting of *M. smegmatis* SmESAT-6, polyclonal antibodies were raised against the N-terminal 20-aa plus a C-terminal cysteine (Covance Research Products, Inc.), and the antibody was affinity purified using the Sulfolink kit (Pierce Biotechnology). The antibodies against KatG and MspA were kind gifts of Dr. S. Cole and Dr. M. Niederweis, respectively. The antibodies against HA (HA.11) and myc (9E10) were both kind gifts of Dr. J. M. Bishop.

Construction of *snm* complementation plasmids for localization studies.

Each of the *snm5*, *snm7*, and *mycP1* genes were amplified by PCR from wild-type *M. smegmatis* genomic DNA and sequenced. The *Sn*m5 complementation vector (pSEC69) was derived from pMJ31, a vector with the *groEL* promoter and an in-frame C-terminal myc tag (Chapter 3, Table 1). To create pSEC69, we exchanged the pMJ31 *groEL* promoter with the 396 bp region 5' of the *Sm3864* gene (the closest putative promoter in the 5' region of the *snm* region). The *snm5* coding region was cloned into pSEC69, and the *Sm3864* promoter, *snm5* coding region, and C-terminal *myc* tag was subsequently subcloned into the pMV306.kan vector. Besides conferring

Abstract: The purpose of this study was to determine the effect of a 12-week resistance training program on the muscle strength and power of the lower extremities in healthy young adults. The subjects were 15 male and 15 female college students, aged 18-25 years, who were randomly assigned to either a resistance training group or a control group. The resistance training group performed a 12-week program of resistance training, while the control group performed no exercise. The subjects were tested at baseline and at 12 weeks for muscle strength and power. The results showed that the resistance training group had significantly greater increases in muscle strength and power than the control group. The findings suggest that a 12-week resistance training program is effective in improving muscle strength and power in healthy young adults.

(Musical notation for "The Rose Tree")

kanamycin-resistance, this vector also drives integration at the *attB* site in the genome, further insuring endogenous *Snm5* expression levels.

We attempted to clone *Snm6* in a similar fashion, replacing its *groEL* promoter with the *Sm3864* promoter, but it did not complement the *SmESAT-6* export defect of the *snm6* mutant. This may indicate that *Snm6* expression, similar to *Snm7* and mycosin-1, is strictly regulated, and we have merely selected the inappropriate promoter. Other groups have predicted that an interior promoter exists in the *snm* region, controlling the *Sm3867-snm2* expression (Pym et al., 2003). This region is only 39 nucleotides and 15 nucleotides in length in *M. tuberculosis* and *M. smegmatis*, respectively, which might indicate that this promoter may overlap with the *snm5* transcript. If true, this might also explain our difficulties in complementing *snm1* or *snm2* with a variety of complementation strategies.

In order to generate the *snm7* and *mycP1* constructs, we subcloned the regions of pSEC80 and pSEC82 (described in Materials and Methods of Chapter 3) containing the endogenous *mycP1* promoter, gene coding region (*snm7* and *mycP1* in pSEC80 and 82, respectively), and C-terminal 2xHA tag into the pMV306.kan integrating vector. All constructs were transformed into their appropriate knock-out strain and tested for their ability to complement the *SmESAT-6* secretion defect. As opposed to Δsnm cells transformed with pMV306.kan alone, the mutants expressing tagged *Snm* proteins were capable of *SmESAT-6* secretion, though tagged mycosin-1 may only be partially functional.

[illegible]

Surface-localized cell wall protein extraction

Cells were cultured as above, and the first PBS wash was collected for Western blot analysis. The washed cell pellet was then resuspended in PBS + 0.2% Tween-80, and vortexed twice at maximum speed for 10 seconds. The cells were pelleted with a low-speed spin (3,000 x g for 10 minutes), and the supernatant (representing those proteins released from the cell wall surface) and PBS-alone wash were filtered using a 0.22µm syringe filter. Samples were normalized for original cell volume and analyzed by SDS-PAGE and Western blot as above. Additionally, we tested various amounts and types of detergents (0.02% and 2% Tween-80 and 2% SDS) and various disruption methods (tube inversion, probe sonication, nutation at 4°C for 1 hour after vortexing), none of which improved the efficiency of cell wall surface protein release without compromising cell integrity (judged by KatG release).

[illegible]

$\frac{d}{dt} \left(\frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}$

Table 1. Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics
<i>mc</i> ² 155	wild-type
<i>mc</i> ² 1 (ATCC607)	wild-type isolate (<i>mc</i> ² 155 parental strain)
ATCC19420	clinical isolate
SCM17	$\Delta snm5$
SCM25	$\Delta Sm3868$
SCM29	$\Delta snm6$
SCM30	$\Delta snm1$
SCM26	$\Delta snm2$
SCM27	$\Delta esxB$
SCM31	$\Delta esxA$
SCM32	$\Delta snm4$
SCM28	$\Delta snm7$
SCM21	$\Delta mycP1$
SCM68	<i>mc</i> ² 155 + pMV306.kan
SCM156	$\Delta snm5$ + pSEC116.int
SCM158	$\Delta Sm3882c$ + pSEC80.int
SCM159	$\Delta mycP1$ + pSEC82.int
MJ10	wild-type + pMJ10
Plasmids	
pMV306.kan	Kan ^R , integrating
pSEC69	<i>Sm3864</i> promoter, C-terminal in-frame <i>myc</i> tag
pSEC116.int	<i>Sm3864</i> promoter, <i>snm5-myc</i> , Kan ^R , integrating
pSEC80.int	<i>mycP1</i> promoter, <i>Sm3882c-2xHA</i> , Kan ^R , integrating
pSEC82.int	<i>mycP1</i> promoter, <i>mycP1-2xHA</i> , Kan ^R , integrating
pMJ10	<i>groEL</i> promoter, <i>mmpL7-2xHA</i> , Kan ^R , episomal

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 二、 $\frac{1}{x^3} = x^{-3}$, $\therefore \frac{d}{dx} x^{-3} = -3x^{-4} = -\frac{3}{x^4}$
 三、 $\frac{1}{x^4} = x^{-4}$, $\therefore \frac{d}{dx} x^{-4} = -4x^{-5} = -\frac{4}{x^5}$
 四、 $\frac{1}{x^5} = x^{-5}$, $\therefore \frac{d}{dx} x^{-5} = -5x^{-6} = -\frac{5}{x^6}$
 五、 $\frac{1}{x^6} = x^{-6}$, $\therefore \frac{d}{dx} x^{-6} = -6x^{-7} = -\frac{6}{x^7}$
 六、 $\frac{1}{x^7} = x^{-7}$, $\therefore \frac{d}{dx} x^{-7} = -7x^{-8} = -\frac{7}{x^8}$
 七、 $\frac{1}{x^8} = x^{-8}$, $\therefore \frac{d}{dx} x^{-8} = -8x^{-9} = -\frac{8}{x^9}$
 八、 $\frac{1}{x^9} = x^{-9}$, $\therefore \frac{d}{dx} x^{-9} = -9x^{-10} = -\frac{9}{x^{10}}$
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 六十、 $\frac{1}{x^{61}} = x^{-61}$, $\therefore \frac{d}{dx} x^{-61} = -61x^{-62} = -\frac{61}{x^{62}}$
 六十一、 $\frac{1}{x^{62}} = x^{-62}$, $\therefore \frac{d}{dx} x^{-62$

[illegible]

Figure 1. Wild-type *M. smegmatis* mc²155 morphology by scanning electron microscopy.

(A-D) Wild-type *M. smegmatis* mc²155 were grown on Sauton's or 7H10 plates, scraped onto filters, coated with carbon/platinum, and analyzed by scanning electron microscopy. Panels A-D are representative images taken at various magnification. The extracellular filaments or fimbriae (EFs) are denoted with asterisks and whether they mediate cell-cell (**) or cell-substrate (*) contacts is denoted. A filament several microns in length is noted in panel A (#).

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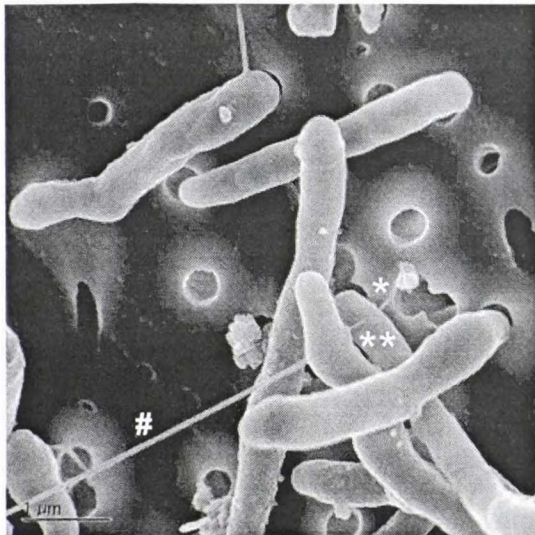
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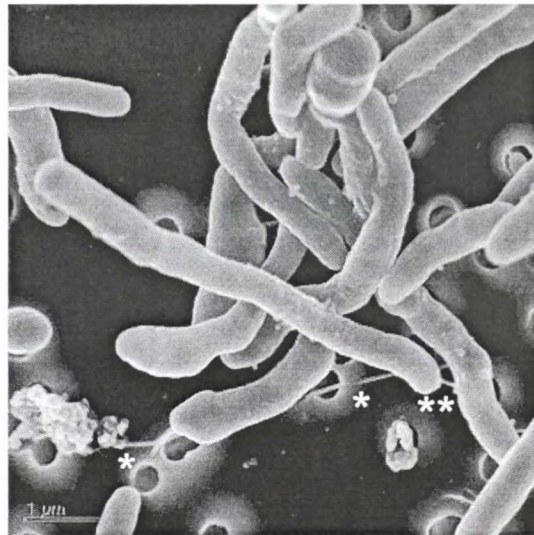
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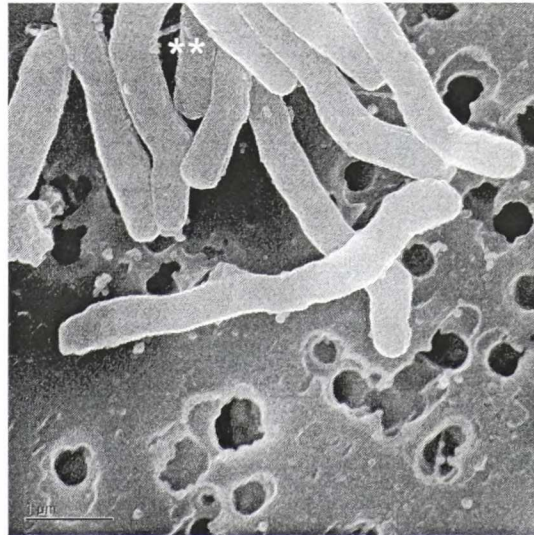
B



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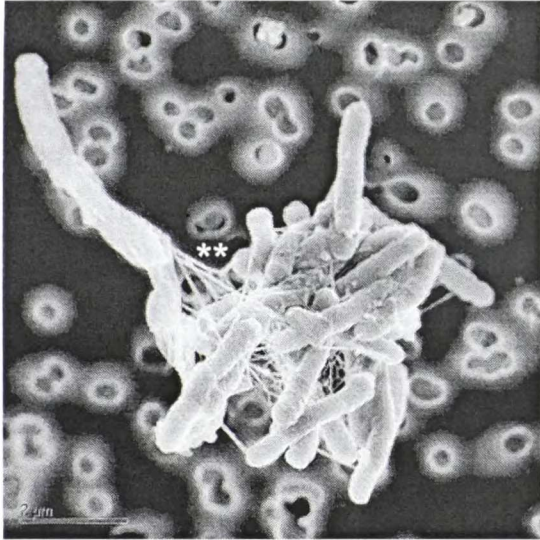
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Figure 2. *M. smegmatis* mc²155 parental strain (mc²1) morphology by scanning electron microscopy.

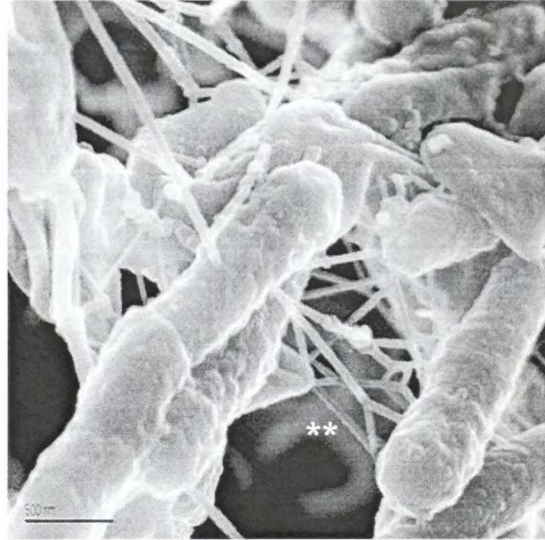
(A-D) The mc²155 parental strain (mc²1 or ATCC607) was grown on Sauton's or 7H10 plates, scraped onto filters, coated with carbon/platinum, and analyzed by scanning electron microscopy. Panels A-D are representative images taken at various magnification. The EFs are denoted with asterisks and whether they mediate cell-cell (**) or cell-substrate (*) contacts is denoted.

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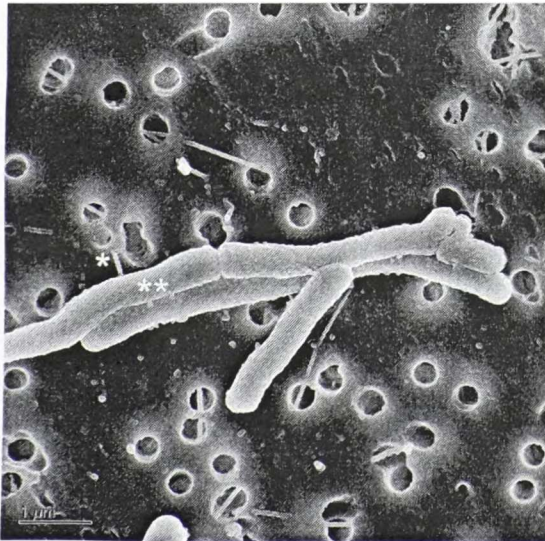
A



B



C



D



Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

Fig. 15

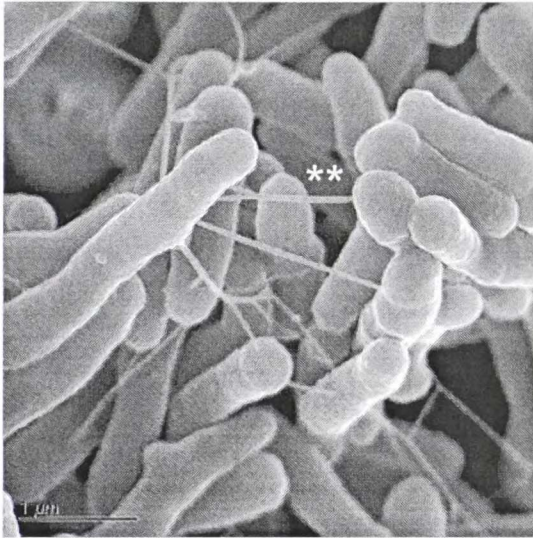
Figure 3. *M. smegmatis* clinical isolate (ATCC19420) morphology by scanning electron microscopy.

(A-D) *M. smegmatis* wild-type clinical isolate (ATCC19420) was grown on Sauton's or 7H10 plates, scraped onto filters, coated with carbon/platinum, and analyzed by scanning electron microscopy. Panels A-D are representative images taken at various magnification. The EFs are denoted with asterisks and whether they mediate cell-cell (**) or cell-substrate (*) contacts is denoted.

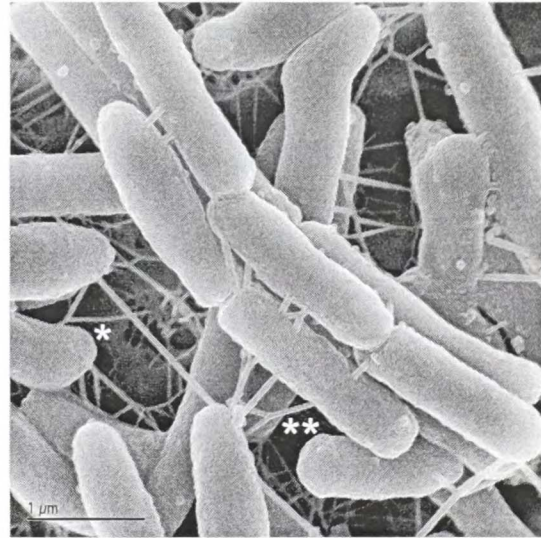
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Handwritten text in a cursive script, likely a letter or document. The text is written in dark ink on a light-colored paper. The script is dense and fills the left side of the page.

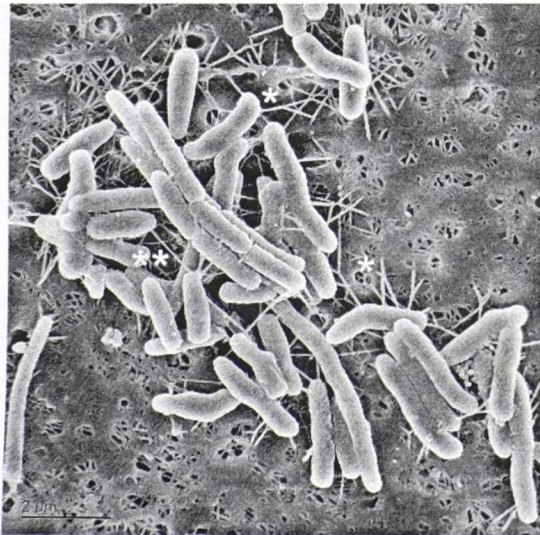
A



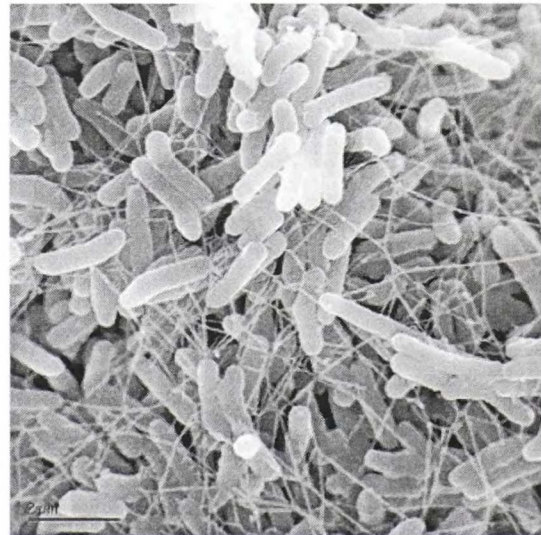
B



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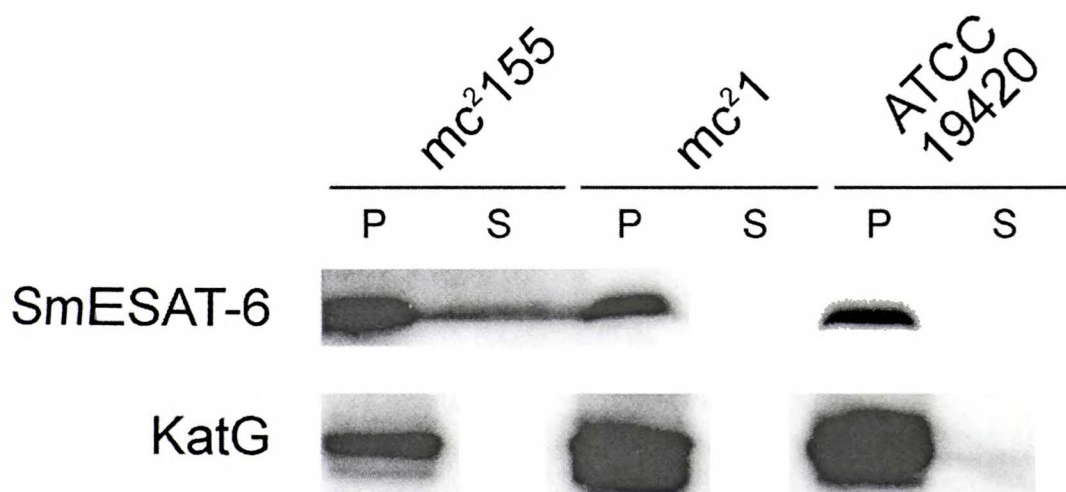
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Figure 4: Comparison of SmESAT-6 secretion from *M. smegmatis* clinical isolates

Various wild-type *M. smegmatis* strains (mc²155, mc²1, and ATCC19420) were grown to late-log phase in Sauton's media without Tween-80, fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blot for the presence of SmESAT-6 and KatG.

[illegible]

The Rose Tree



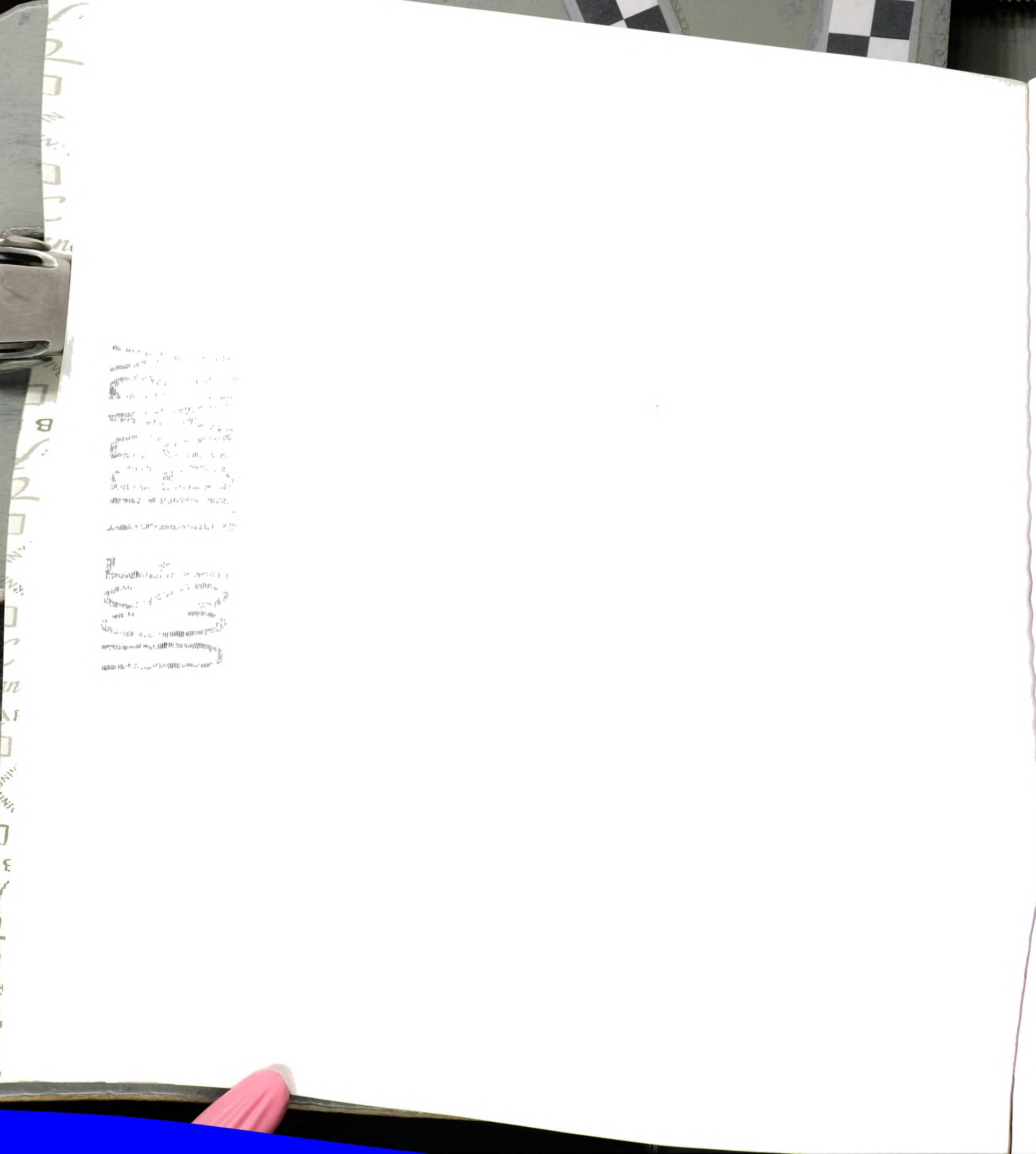
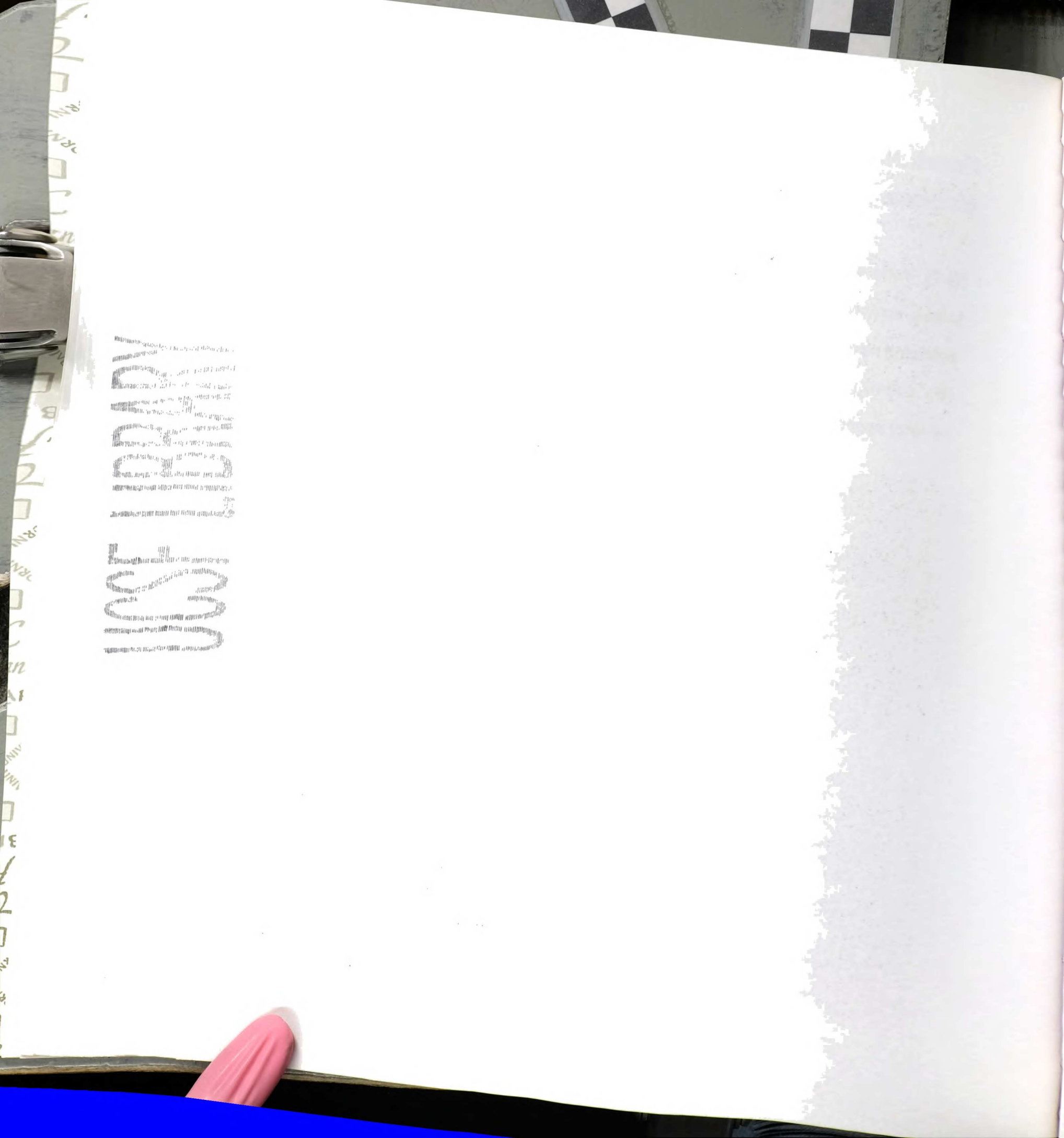
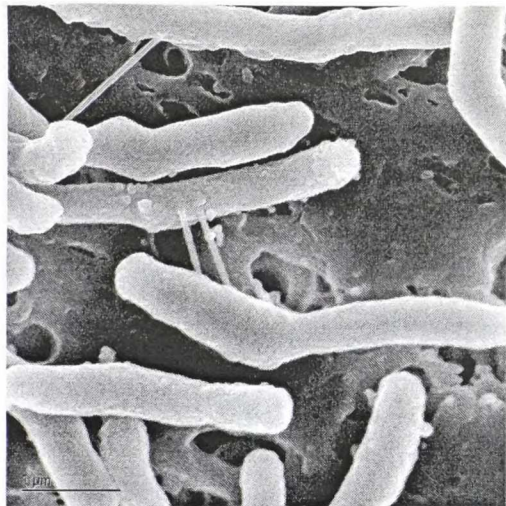


Figure 5. *M. smegmatis* *snm* and *esx* mutant morphology by scanning electron microscopy.

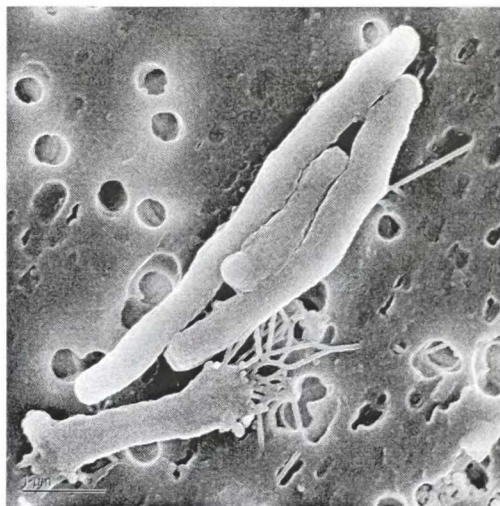
(A-F) Various *snm* and *esx* mutant cells were grown on Sauton's or 7H10 plates, scraped onto filters, coated with carbon/platinum, and analyzed by scanning electron microscopy. The strains depicted are $\Delta mycP1$ (**A**), $\Delta Sm3868$ (**B**), $\Delta snm2$ (**C**), $\Delta snm7$ (**D**), $\Delta snm5$ (**E**), $\Delta esxB$ (**F**). Asterisks in panel E denote several apparent "mini-cells," and asterisks in panel F denote a swollen cell.



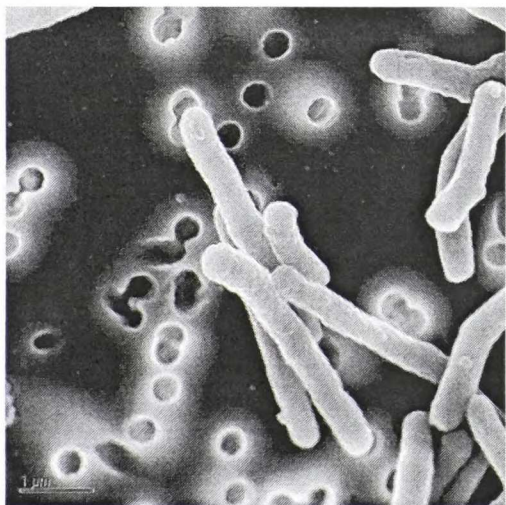
A



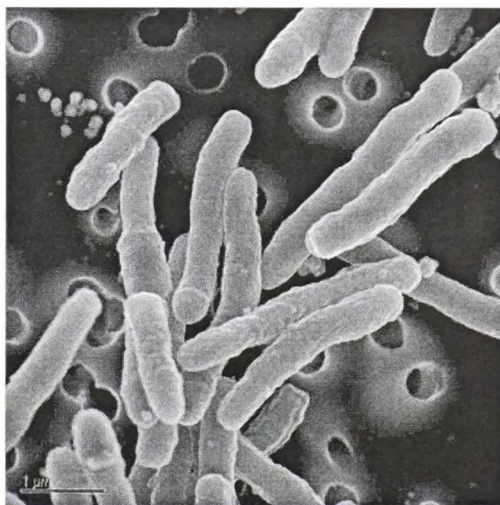
B



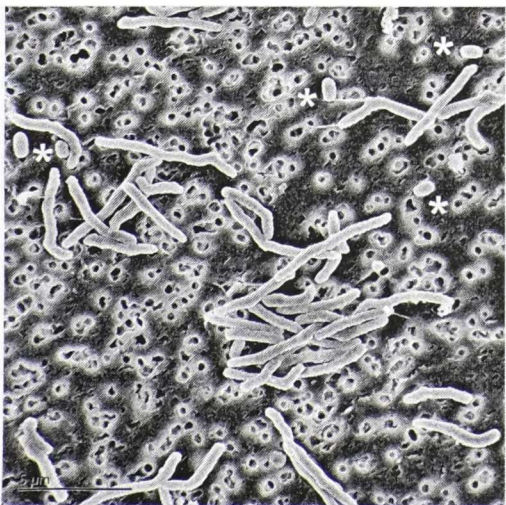
C



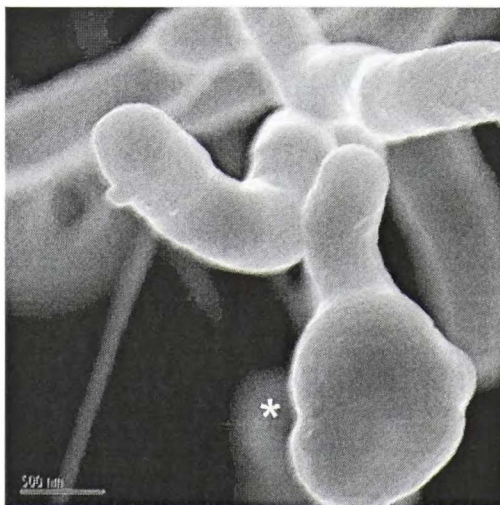
D



E



F



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Figure 6. Subcellular fractionation of wild-type *M. smegmatis* and media-dependent MspA export.

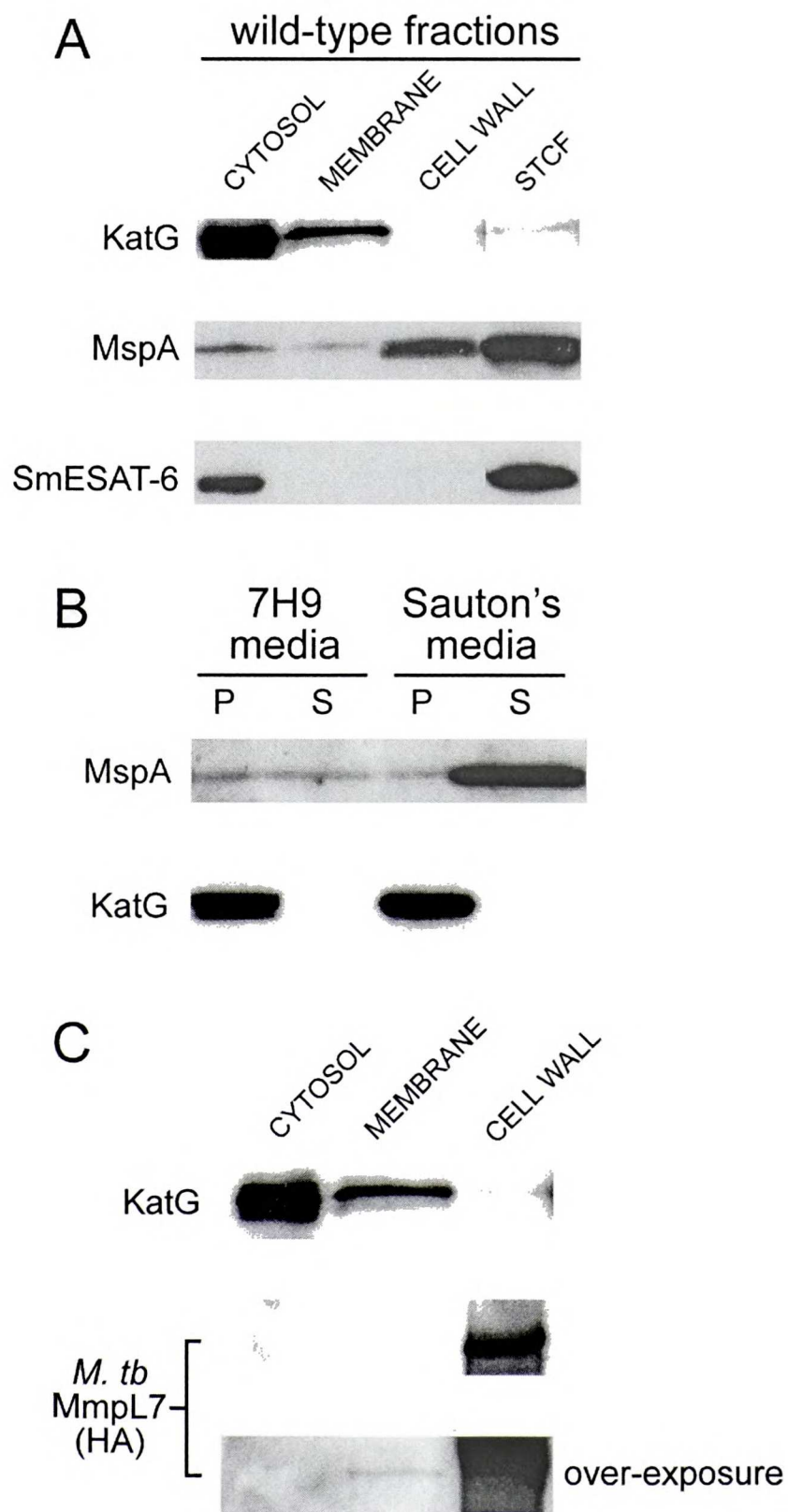
A) Wild-type cells were grown to late-log phase in Sauton's media, and the short-term culture filtrate (S) fraction was collected as described previously. The cells were washed with PBS then disrupted using a beadbeater. Unbroken cells were removed by four low-speed spins, and the resulting lysate was centrifuged at 35,000 x g to pellet the cell wall fraction. The supernatant was subsequently centrifuged at 100,000 x g to pellet the membrane fraction from the cytosolic supernatant. The protein levels in each fraction were quantified by MicroBCA, and equivalent protein amounts were loaded on an SDS-PAGE gel. The relative levels of the indicated proteins were determined by Western blot analysis.

B) Wild-type cells were grown to late-log phase in 7H9 or Sauton's media (without Tween-80), fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blot for the presence of MspA and KatG.

C) Wild-type cells expressing the exogenous *M. tb* MmpL7 transmembrane protein (tagged with HA at its C-terminus) were fractionated as described in **A)**. The relative levels of MmpL7 were determined by probing each fraction for HA. The bottom two panels are different exposures of the same MmpL7-HA Western blot.

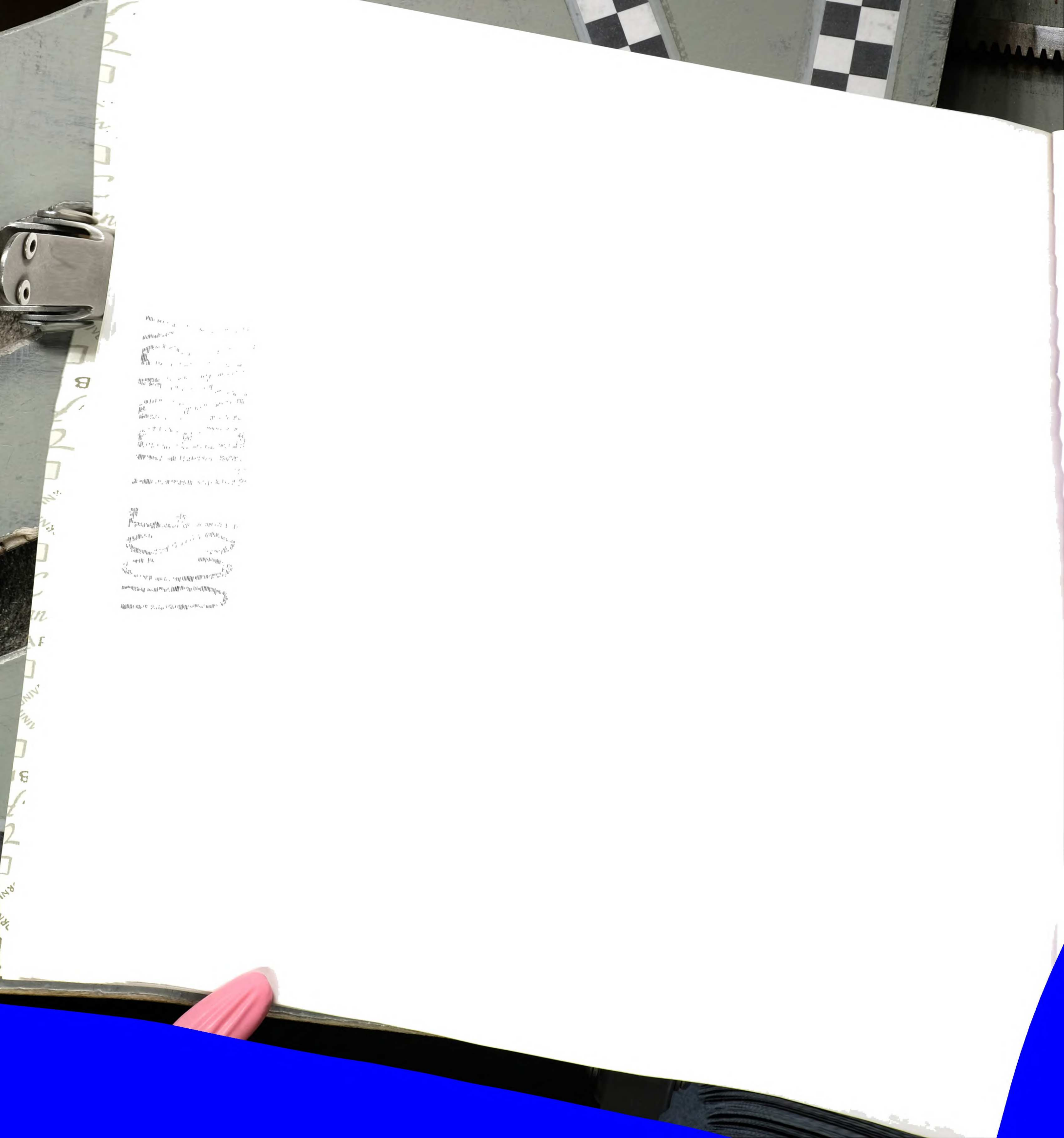
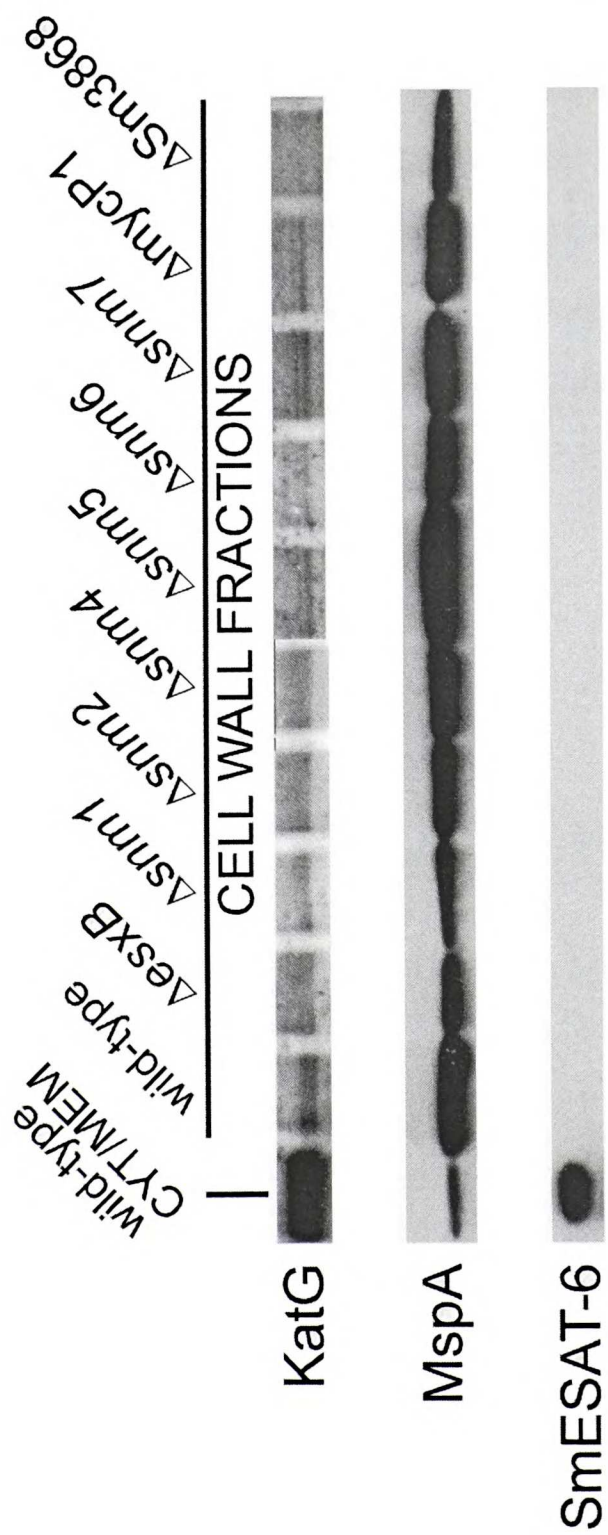


Figure 7. Subcellular fractionation of *snm* and *esx* mutants.

Wild-type and *snm/esx* mutant cells were grown to late-log phase in Sauton's media, and the cells were fractionated and analyzed as described previously.

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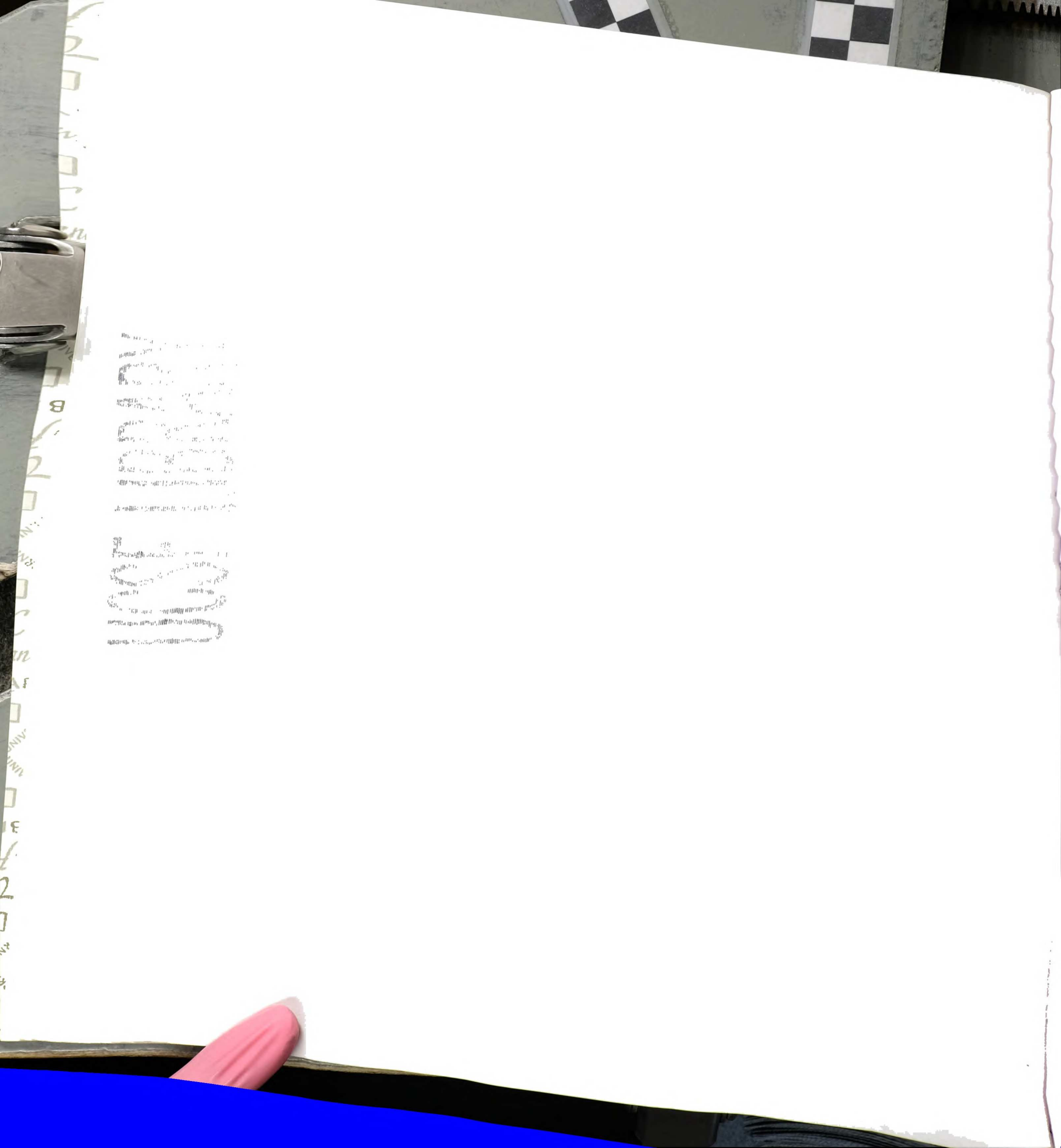
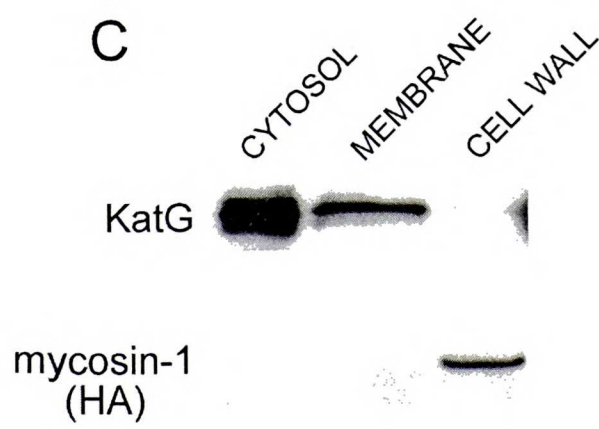
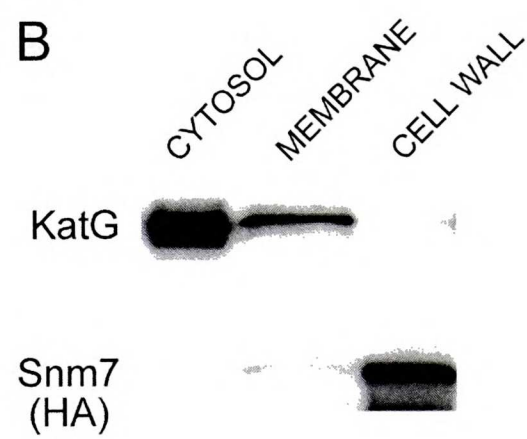
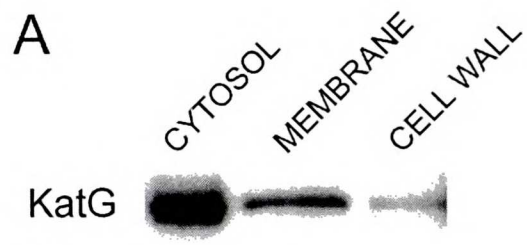


Figure 8. Subcellular fractionation of the Snm secretory apparatus.

Three *snm* mutants (*snm5*, *snm7*, and *mycP1* knock-outs) were complemented with integrating plasmids expressing epitope tagged versions of each Snm protein from their endogenous promoter. These cells were cultured and fractionated as described previously, and probing the fractions with antibodies to their appropriate epitope tag (either myc or HA) revealed the enrichment of each Snm protein in each cell fraction.

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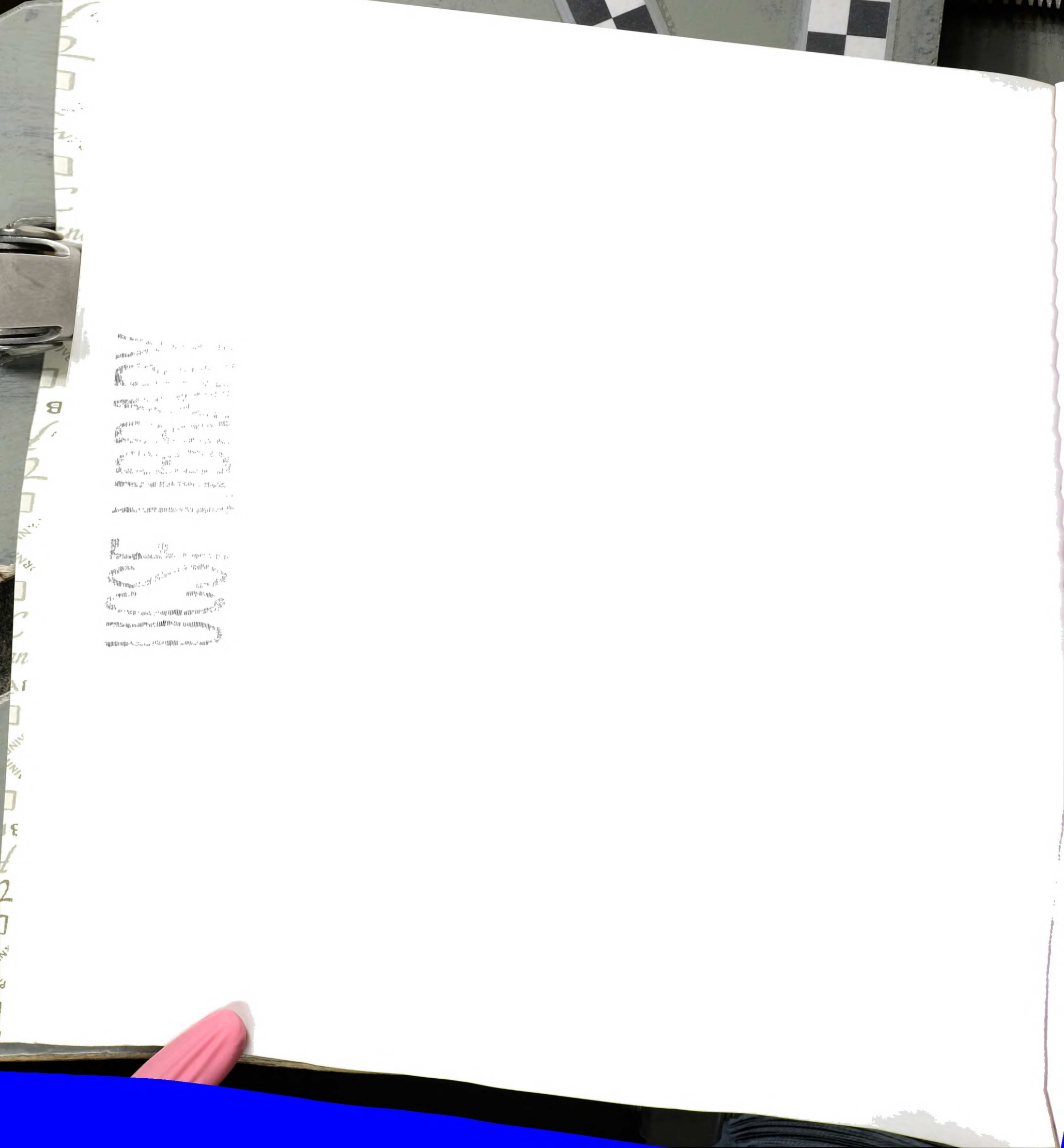


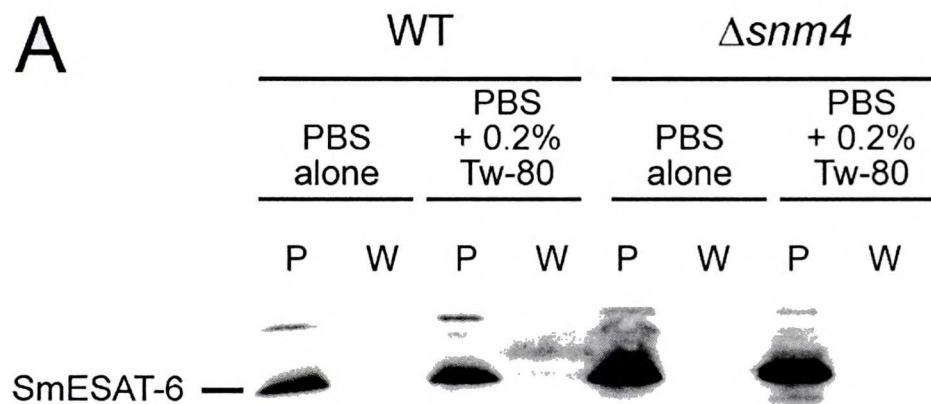
Figure 9. Surface-exposed proteins in wild-type and *snm* mutant *M. smegmatis*.

A) and B) Wild-type and mutant cells were grown to late-log phase in Sauton's media, and the cells were washed once with PBS. The surface-exposed proteins were extracted by resuspending the cells in PBS + 0.2% Tween-80 and briefly vortexing. The cells were removed by centrifugation, and both the PBS and PBS + 0.2% Tween-80 washes (W) were syringe filtered. Proteins were purified from all fractions using TCA precipitation, and, after normalizing for original culture volume, samples were analyzed by SDS-PAGE and Western blot.

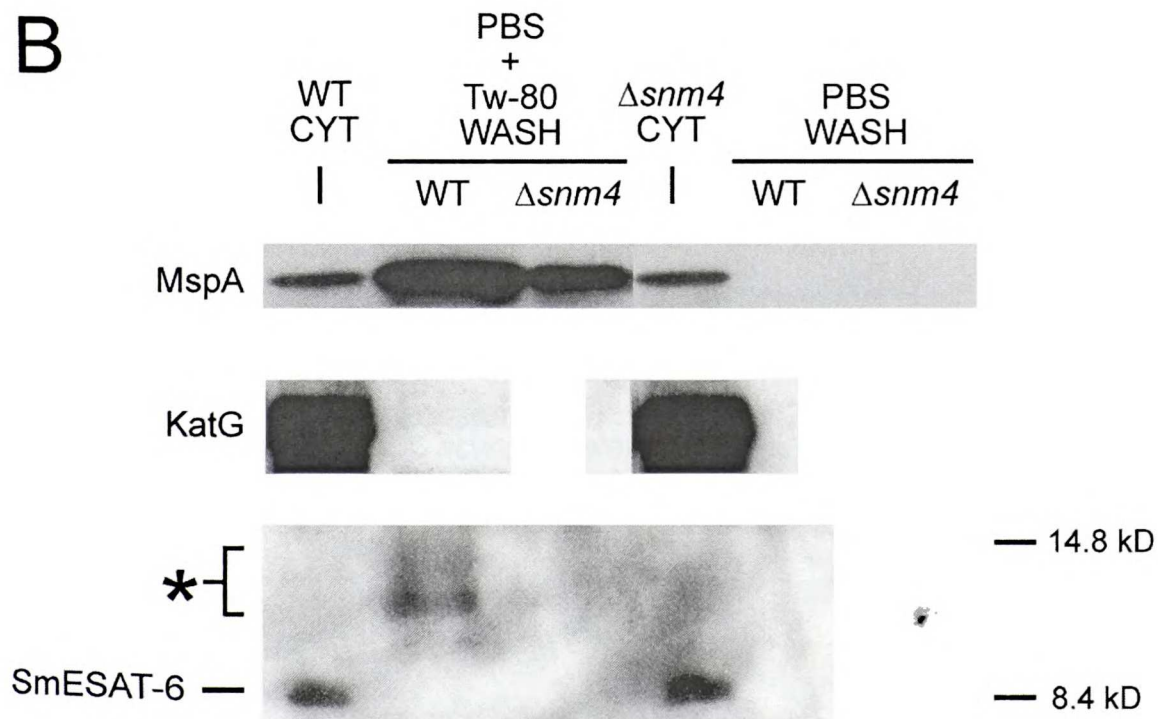
Handwritten musical notation on a staff, including notes and rests.

Handwritten musical notation on a staff, including notes and rests.

A



B



$$P_{11} = \frac{1}{2} \left(\frac{1}{2} + \frac{1}{2} \right) = \frac{1}{2}$$

$$P_{12} = \frac{1}{2} \left(\frac{1}{2} - \frac{1}{2} \right) = 0$$

$$P_{21} = \frac{1}{2} \left(\frac{1}{2} + \frac{1}{2} \right) = \frac{1}{2}$$

$$P_{22} = \frac{1}{2} \left(\frac{1}{2} - \frac{1}{2} \right) = 0$$

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Figure 10. Alignment of tight-adherence pathway homologues in *Actinobacillus actinomycetemcomitans* and mycobacterial species.

A) The tight-adherence pathway pilin subunits, Flp1 and Flp2, were aligned with the proposed homologues from *M. tuberculosis* and *M. smegmatis*.

Sequence information for *M. tuberculosis*, *M. smegmatis*, and *A.*

actinomycetemcomitans were obtained from Tuberculoist

(<http://genolist.pasteur.fr/TubercuList/>), TIGR ([http://www.tigr.org/tigr-](http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gms)

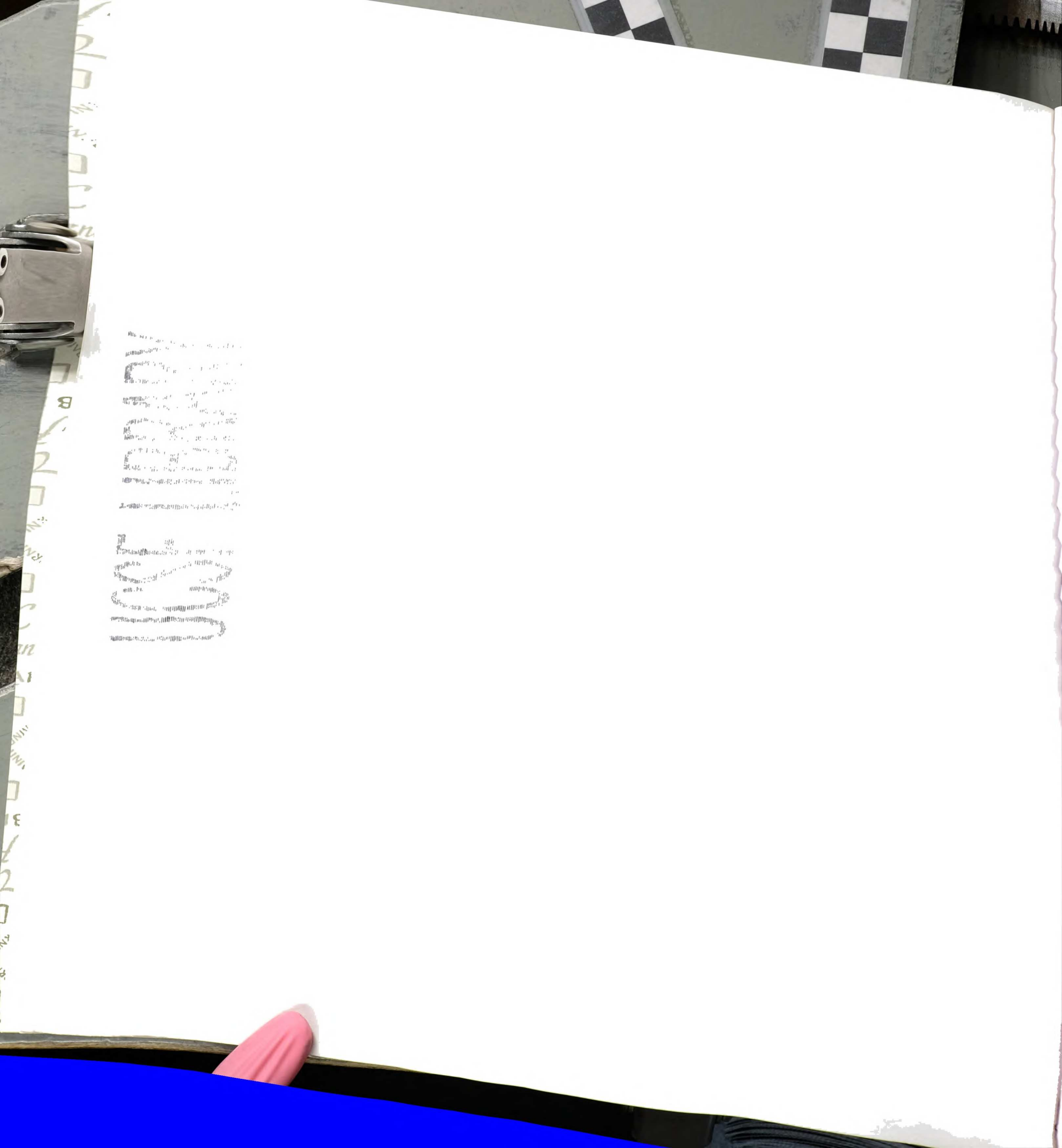
[scripts/CMR2/GenomePage3.spl?database=gms](http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gms)), and NCBI

(<http://www.ncbi.nlm.nih.gov/>), respectively. Alignments were performed using

CLUSTALW (<http://www.ebi.ac.uk/clustalw/>). Abbreviations: Mtb: *M.*

tuberculosis; Msm: *M. smegmatis*; Aa: *A. actinomycetemcomitans*. **B)** The

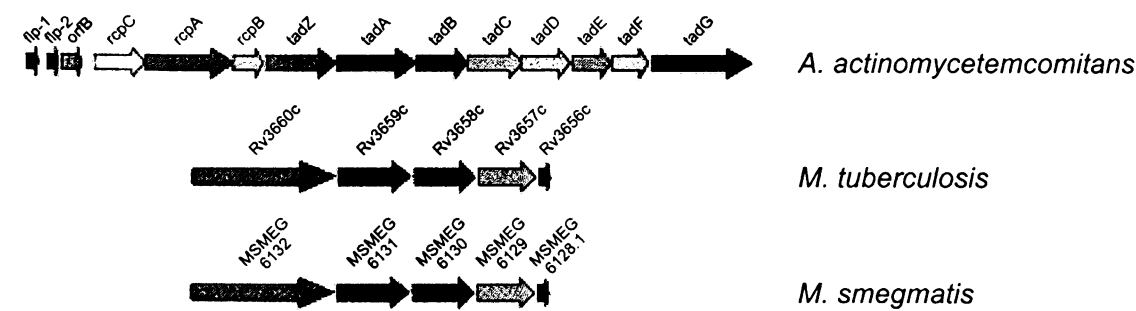
Tad genomic loci, including the surrounding genes encoding the dedicated pilin secretion machinery, from *A. actinomycetemcomitans* was compared to the loci from *M. tuberculosis* and *M. smegmatis*.



A)

Flp-1	Mtb	---	MLVITMFRVLVARM	TALAVDESGMSTVEYAIGTIAAAAFGAILYTVVTG--	DSIVSA	55
Flp-1	Msm	----	VIQVFRRVQARM	ALLAVDDSGMSTVEYALGTVAAAAFGAILYTVVTG--	DSVVNA	53
Flp-1	Aa		MLNTLT	TTKAYIKAAEAIRSFRENQAGVTAIEYGLIAIAVAVLIVAVFYSNNGFIANLQSK		60
Flp-2	Aa		MMD-LLD	YFYRQVVFFSYKFYRNRQGITSVEYGLIAVAIAVFVVAVLVGDNSFISALSGK		59
			:	:	:	:
Flp-1	Mtb		LNRIIGRALSTKV----			68
Flp-1	Msm		LSNIISRALNTSV----			66
Flp-1	Aa		FNSLASTVNSANVTK--			75
Flp-2	Aa		FSDLATTVSGAIVSKSS			76
			:	:	:	:

B)



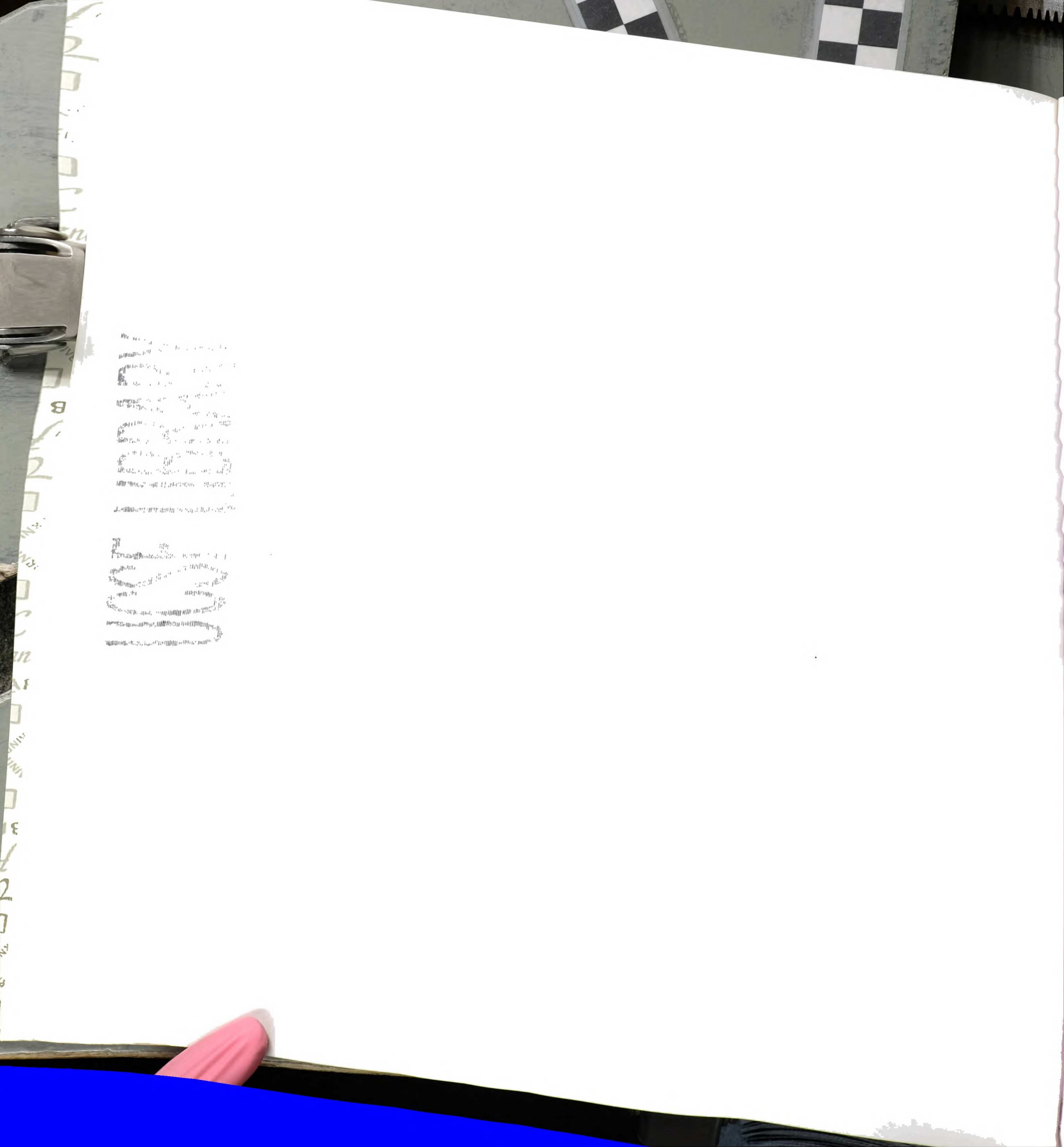
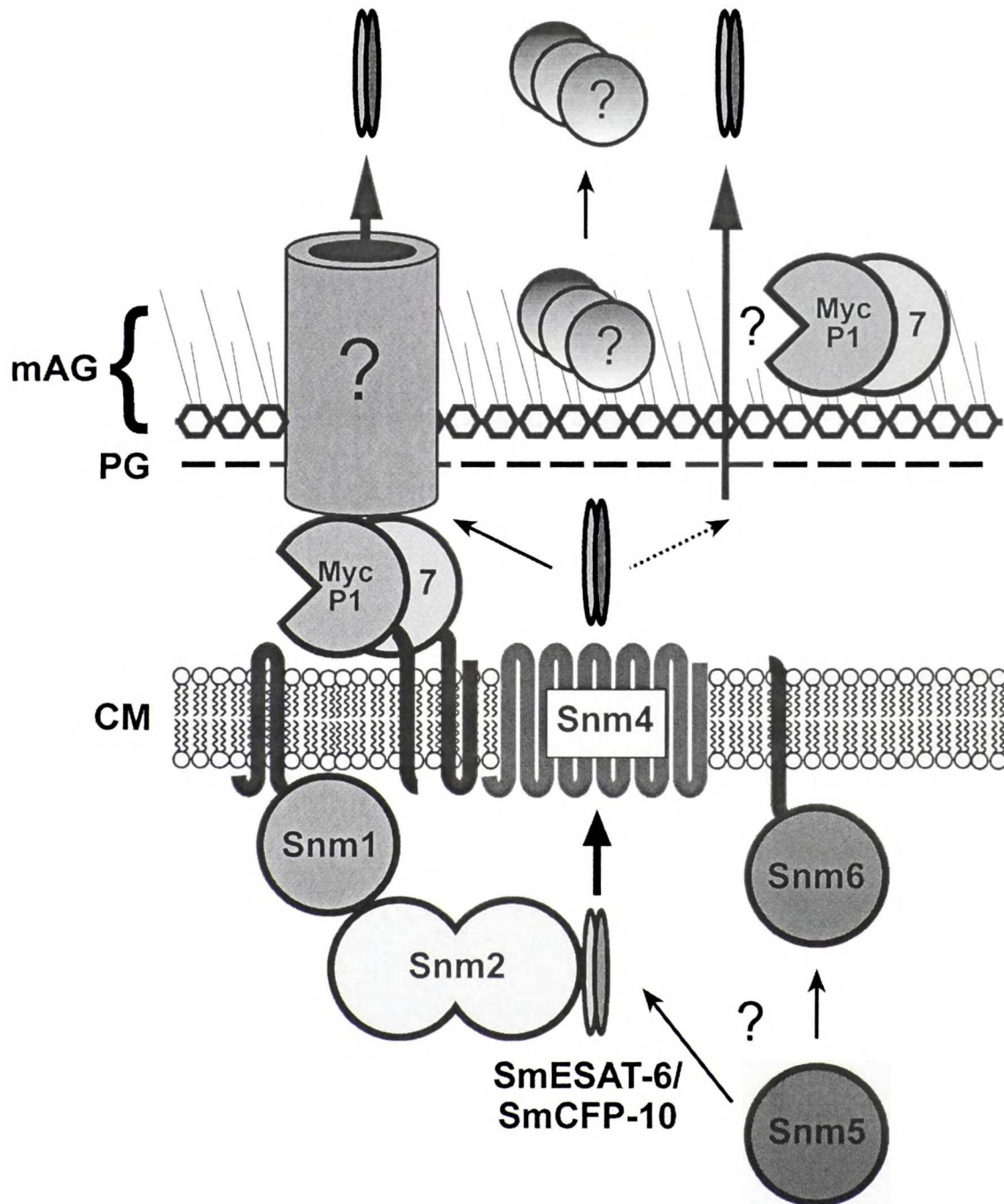


Figure 11. Current model of the Snm secretion pathway in *M. smegmatis*.

See *Discussion* for details of the model. CM, cytoplasmic membrane; PG, peptidoglycan layer; mAG, mycolyl arabinogalactan layer. Transmembrane domains were predicted by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

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Chapter 5

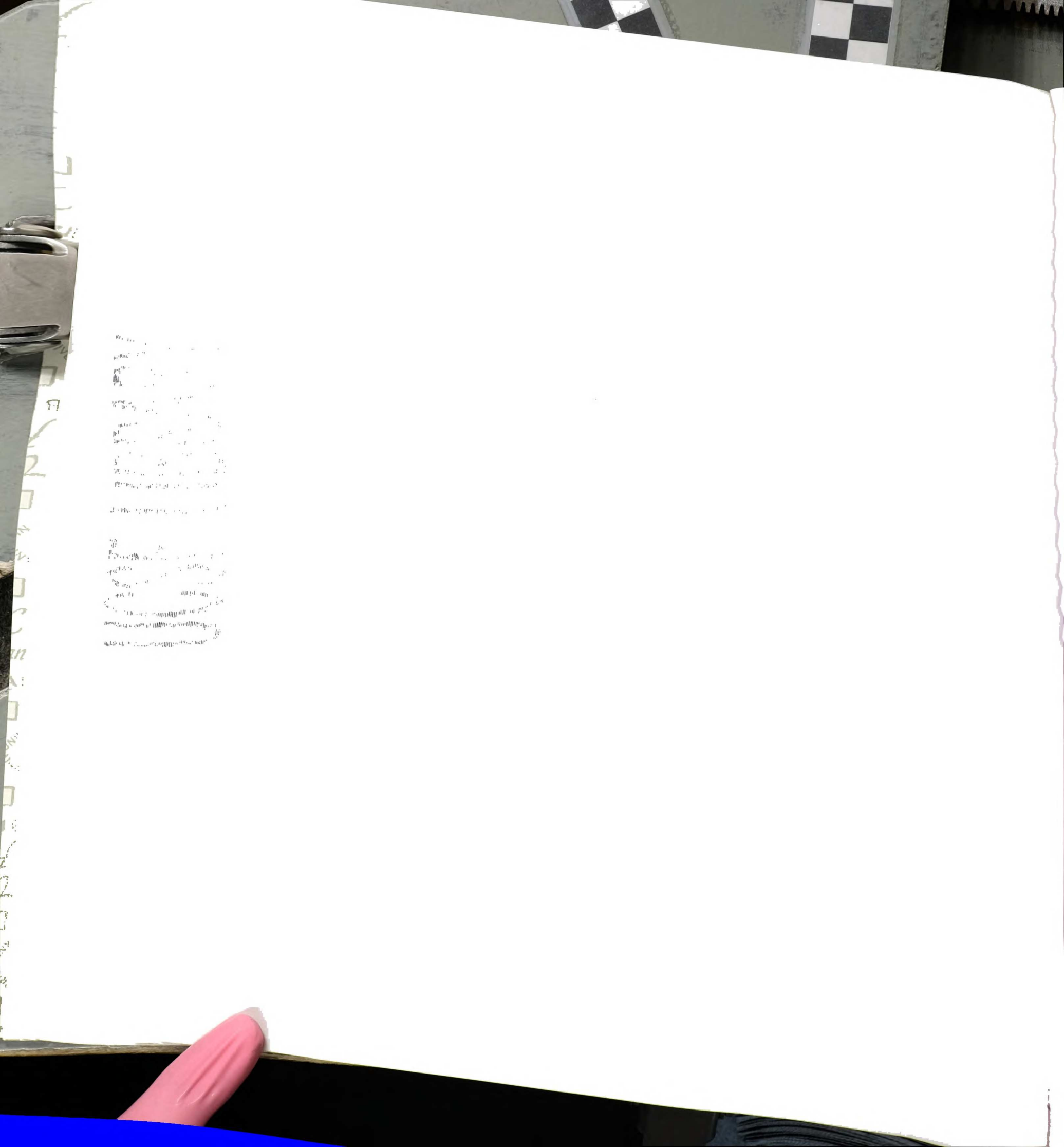
Conclusions and Perspectives

1. The first part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are based on the principle of the conservation of energy.

2. The second part of the paper is devoted to a discussion of the general principles of the theory of the structure of the molecule. It is shown that the structure of the molecule is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are based on the principle of the conservation of energy.

Despite exhaustive experimentation since its discovery, genetic manipulation of *M. tuberculosis* is only a recent phenomenon. In fact, the pool of mutants Jeff brought to UCSF represented the first published screen for virulence factors in *M. tuberculosis*. This allowed us to approach experiments with true controls and genetic precision which inevitably complicated interpretations of previous experimenters. Ultimately, our genetic approach in *M. tuberculosis*, and eventually *M. smegmatis*, allowed our lab's rapid discovery of novel virulence mechanisms of mycobacteria. Both the MmpL and Snm virulence pathways involve large families of components, and numerous homologues are encoded in many mycobacterial genomes. This suggests that they mediate absolutely critical (yet poorly understood) pathways in order to aid pathogenesis. While the substrates of these pathways are quite divergent, they represent different strategies to the same end: delivery of virulence factors beyond the the mycobacterial cell wall.

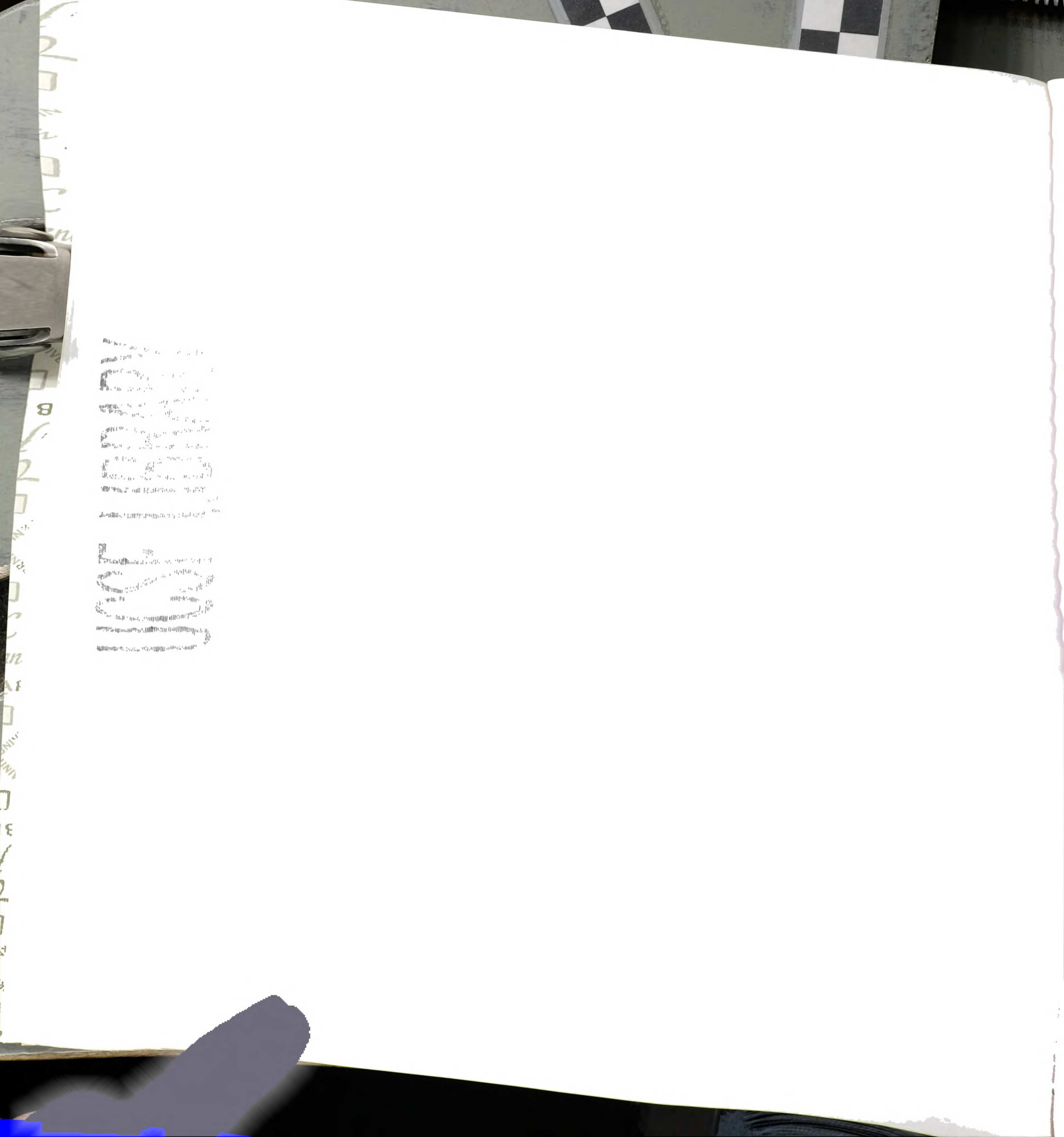
Our early work on the MmpL8 pathways identified the second MmpL family member important for virulence. Using simple biochemical procedures, we implicated this lipid transporter in the biogenesis of sulfolipid, a complex cell wall lipid long thought to function in pathogenesis. An important result of our MmpL8 studies was the discovery of SL₁₂₇₈, an intermediate form of sulfolipid. Using genetic evidence and FT-ICR mass spectrometry, we were able to confidently assign a structure that has subsequently been confirmed by two other groups (Domenech et al., 2004; Gilleron et al., 2004). Interestingly, Gilleron et al independently purified SL₁₂₇₈ as a potent activator of CD8⁺ T cells



in vitro and *in vivo*. They further demonstrated that CD1b, a member of a family of MHC-like molecules in antigen presenting cells whose known antigens are almost exclusively mycobacterial in origin (Moody et al., 2005), is responsible for recognizing and presenting SL₁₂₇₈ to lymphocytes. Their findings reveal a functional *in vivo* interaction between SL₁₂₇₈ and host cells and suggest that host cells have adapted specific mechanisms to recognize a precursor in the SL-1 pathway. While the role of SL-1 *in vivo* remains obscure, these findings suggest that *M. tuberculosis* may tightly regulate SL₁₂₇₈ levels to avoid detection by host cell defenses.

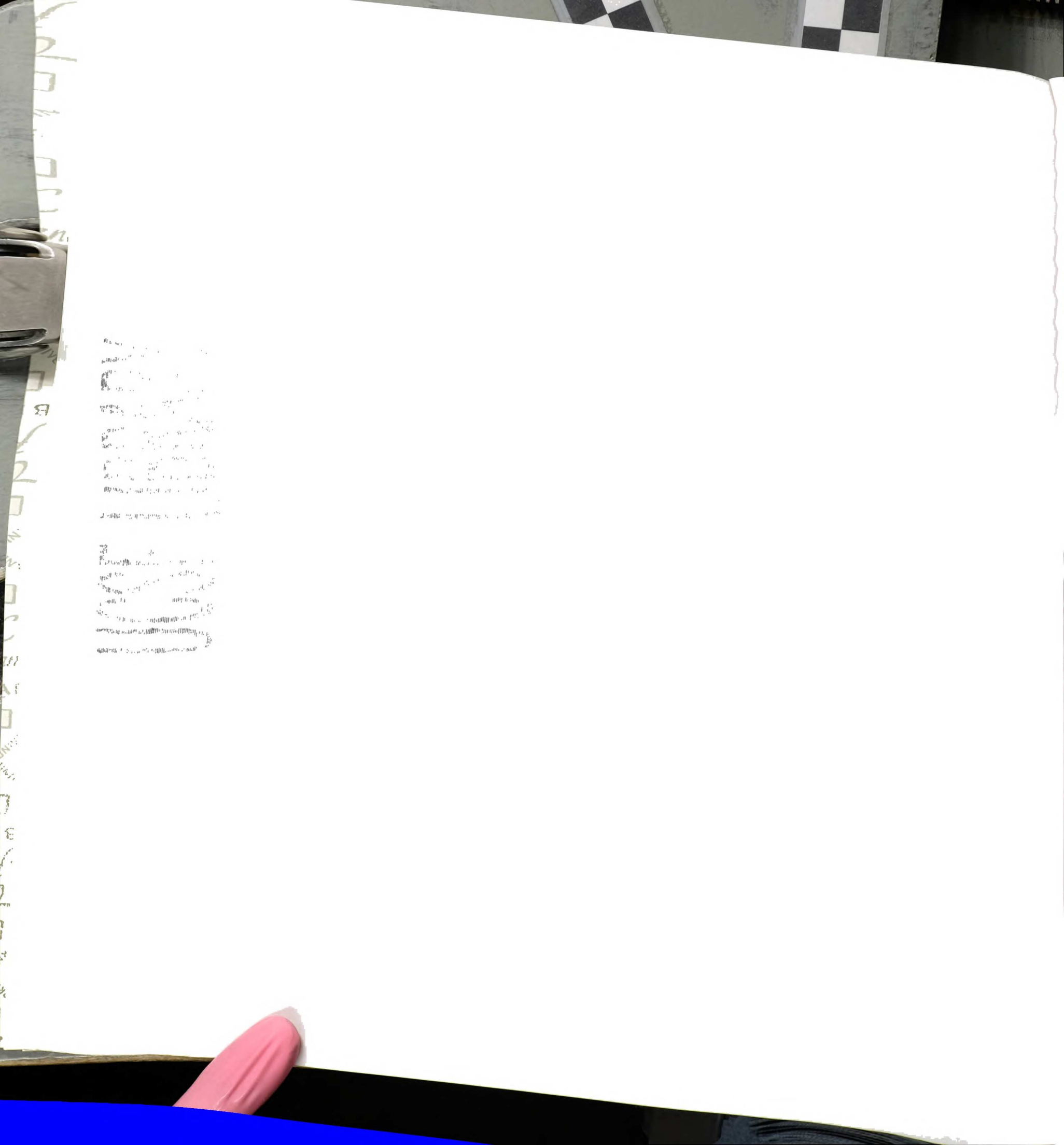
The antigenic nature of SL₁₂₇₈ provides an additional explanation for the attenuation of the $\Delta mmpL8$ strain. While mice lack group I CD1 molecules (CD1a-c), it is possible that mouse CD1d recognizes the accumulated SL₁₂₇₈ in $\Delta mmpL8$, causing the attenuated *in vivo* phenotype. Importantly, further work to characterize the *in vivo* roles of SL-1 and SL₁₂₇₈ should be performed in model organisms possessing the entire CD repertoire. Furthermore, these findings suggest that the $\Delta mmpL8$ *in vivo* growth phenotype might be more dramatic in cells containing all CD1 isoforms, and that the $\Delta mmpL8$ strain might be a promising vaccine candidate.

The surprising SL-1 synthesis defect of $\Delta mmpL8$ cells provided evidence of an emerging paradigm that mycobacterial polyketide synthesis and secretion are coupled. Besides the role of MmpL8 in SL-1 synthesis, our lab discovered a novel interaction between MmpL7 and PpsE, the polyketide synthase responsible for synthesizing the MmpL7 secretion substrate, PDIM (Jain and



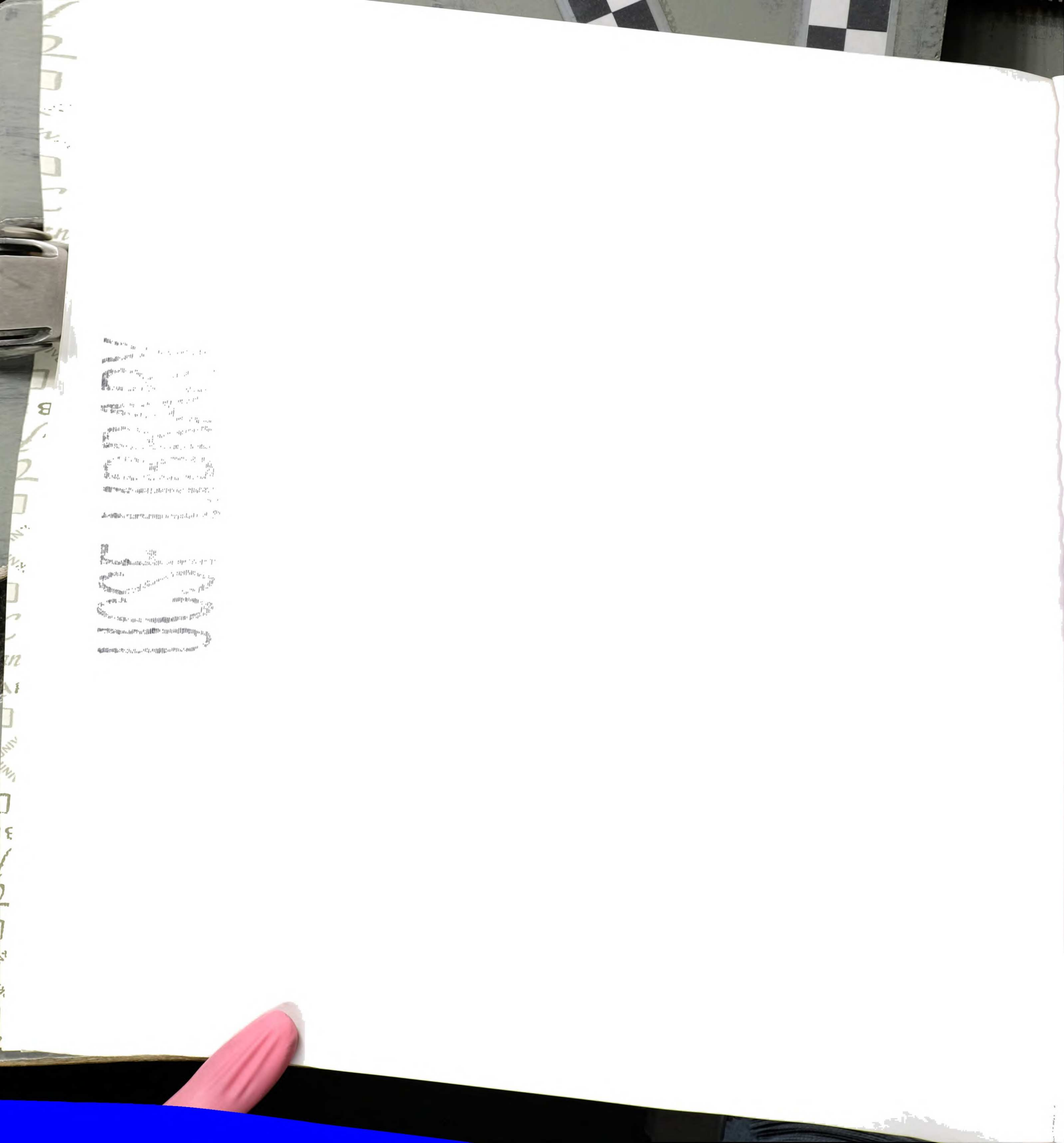
Cox, 2005). Expression of the domain of MmpL7 that interacts with PpsE dramatically reduced PDIM synthesis, and this effect is dependent on specific residues in MmpL7. There are several possible explanations for the synthesis/secretion link. First, the MmpL-Pks interaction may insure efficient targeting for secretion. As *M. tuberculosis* possesses 14 MmpL homologues, these interactions may insure proper secretion regulation and reduce “cross-talk” between the numerous MmpL secretion pathways in the cell. In the case of SL-1, MmpL8 may assist in compartmentalizing the four SL-1 acylation steps, effectively insuring proper SL-1 assembly (see Chapter 2, Figure 6). Further work is needed to determine the purpose of coupling synthesis and secretion, but this feature of the MmpL pathway likely represents a novel adaptation of mycobacteria to aid in the assembly and export of their diverse population of complex lipids and lipoglycans.

As my interests shifted from lipid secretion to protein secretion, our lab published the first work with convincing evidence that a dedicated protein secretory apparatus secreted the two small proteins ESAT-6 and CFP-10. The striking *in vivo* defects of mutants lacking the Snm machine clearly demonstrated the central importance of this pathway for mycobacteria. Furthermore, several genome analysis papers hinted that Snm1-4 might not be the only components of the secretory apparatus. Within the *M. tuberculosis* genome, these authors discovered five copies of the Snm locus with a conserved set of 9 genes surrounding the *esx* and *pe/ppe* genes. Furthermore, they found that the Snm locus was not exclusive to pathogenic *M. tuberculosis*;



the *snm* and *esx* genes are present in non-pathogenic mycobacteria, like *M. smegmatis*, and even other distantly related Gram-positive species, like *B. subtilis*, *C. diphtheriae*, and *S. aureus*. After my work in the BSL-3, I was anxious to develop a more tractable organism to study mycobacteria, and, as the genes are conserved in *M. smegmatis*, this seemed a rapid and simple approach to directly test the hypotheses put forth by the numerous bioinformatics groups. Indeed, using in-frame deletion and complementation of individual predicted *snm* genes, we proved that 8 of the 9 predicted *snm* genes are required for export of SmESAT-6 and SmCFP-10 beyond the cell wall. Remarkably, investigators of the *Snm* pathway in *M. tuberculosis* still lack the comprehensive *snm/esx* knock-out strain collection that we have in *M. smegmatis*.

Moving our experimentation out of the BSL-3 has also allowed us to perform experimentation that is still technically impossible in our BSL-3 laboratory. We developed an improved fractionation protocol to investigate the localization of SmESAT-6 and the *Snm* machine, and our findings conflict with early reports from the Cole group that ESAT-6 is a major component of the *M. tuberculosis* cell wall. This may reflect a true difference between *M. tuberculosis* and *M. smegmatis*, but, as recent work from the Cole group and others has refuted their earlier claims that ESAT-6 resides and functions in the cell wall, we propose that both ESAT-6 and SmESAT-6 are exclusively cytosolic and extracellular. Fractionation studies of our panel of *snm* mutants did not reveal any mutation which trapped SmESAT-6 in the cell wall. While not



conclusive, it is consistent with the hypothesis that the Snm machine provides a continuous conduit to export SmESAT-6 through the thick cell wall layers. Consistent with this hypothesis, we were able to localize epitope-tagged Snm proteins to distinct compartments of *M. smegmatis*. Using subcellular fractionation, we discovered that Snm5 exists in the cytoplasm, while Snm7 and mycosin-1 are major components of the cell wall. Accordingly, we predict that Snm5 functions during the proximal cytosolic translocation events, while Snm7 and mycosin-1 likely function in concert at the distal translocation steps across the peptidoglycan or mycolyl-arabinogalactan layers. Ultimately, our foundation of work in *M. smegmatis* will allow others to not only test these predictions but uncover more mechanistic details about how this complex secretion apparatus assembles and how each component functions to deliver SmESAT-6 to the extracellular milieu.

Furthermore, our characterization of the Snm pathway in *M. smegmatis* uncovered interesting features of SmESAT-6 secretion. Using a new technique to detect surface-localized proteins, we found evidence that additional proteins may utilize the Snm pathway for their delivery to the surface of the cell wall. This is not surprising, as multiple Snm substrates have been found in other species. We also uncovered that the Snm pathway was regulated by the growth media. SmESAT-6 secretion was blocked in 7H9, but not Sauton's media, and, while we were unsuccessful in identifying what precise component(s) promoted or inhibited secretion, our mixing experiments did indicate that 7H9 likely contained one or more ingredients that inhibit secretion.

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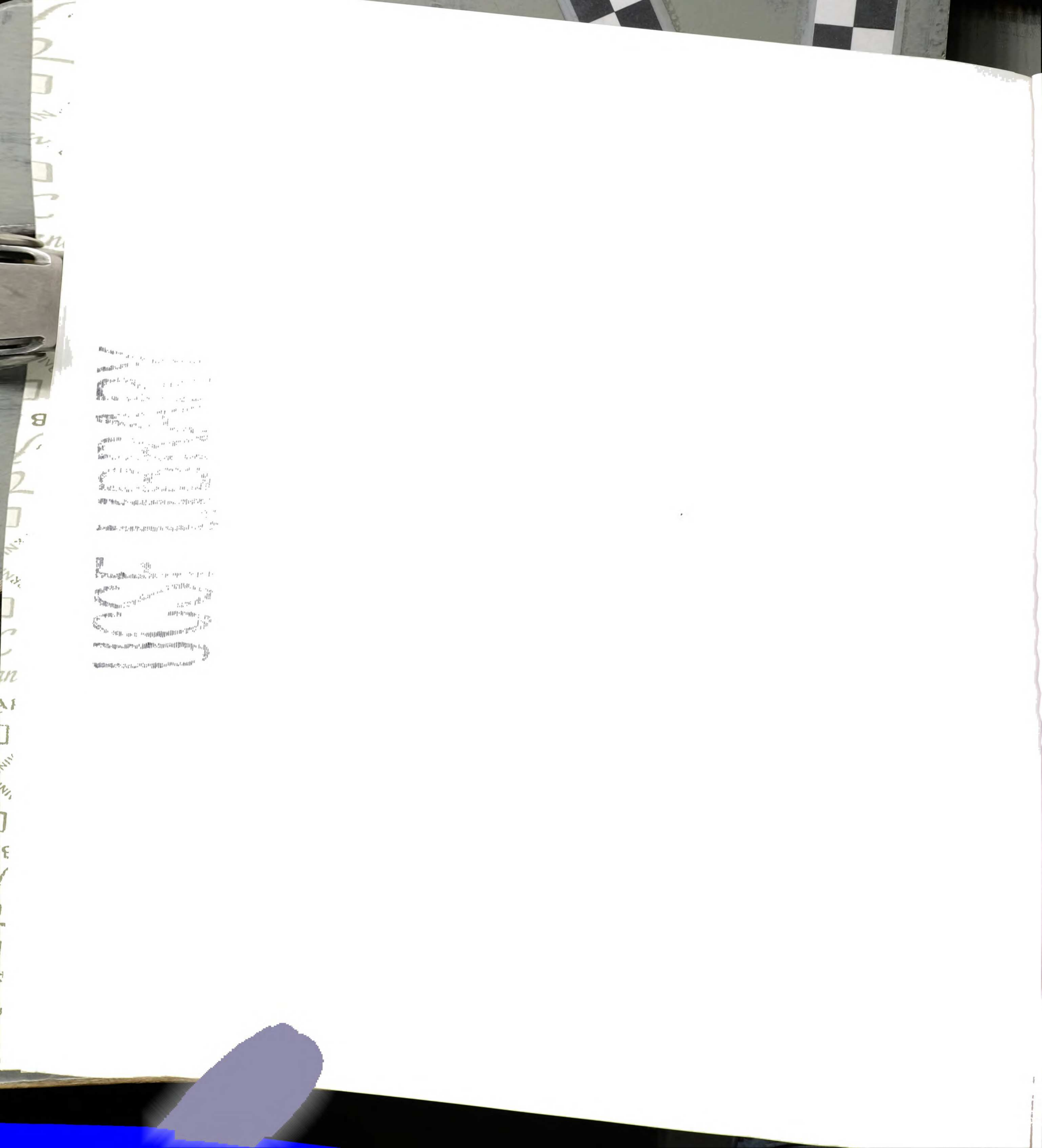
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Because albumin and catalase are present in the *M. tuberculosis* 7H9 media, this experiment has never been performed in *M. tuberculosis*, and future work should address whether this feature of the pathway is conserved between the two species.

In our effort to use new techniques to characterize our panel of *snm* mutants, we also uncovered novel features of *M. smegmatis* that might be relevant to *M. tuberculosis* pathogenesis. We were able to take advantage of the expertise of UC-Berkeley's Electron Microscopy Laboratory to apply the latest EM sample preservation and preparation techniques to mycobacteria, and this approach uncovered striking features of the mycobacterial cell wall. First, we found extensive extracellular filaments or fimbriae (EFs) in multiple strains of *M. smegmatis*. These EFs, which probably consist of several bundled filaments, reach several microns in length where they mediate both cell-to-cell and cell-to-substrate attachments. It is unclear what the function of these EFs might be, but we have found that *M. smegmatis* and *M. tuberculosis* possess genes homologous to a known pathway, termed the tight adherence or Tad pathway, that secretes a bundled filament to attach to substrate surfaces and mediate pathogenesis.

Our SEM approach also uncovered extensive outer-cell wall "blebbing" and extracellular debris in *M. smegmatis*. Due to the rounded appearance of these "blebs" at the surface of the cell wall, which is largely lipidic, we predict that they are, in fact, outer-membrane vesicles (OMVs). OMVs are well studied mechanisms of virulence factor delivery in many Gram-negative bacteria, but

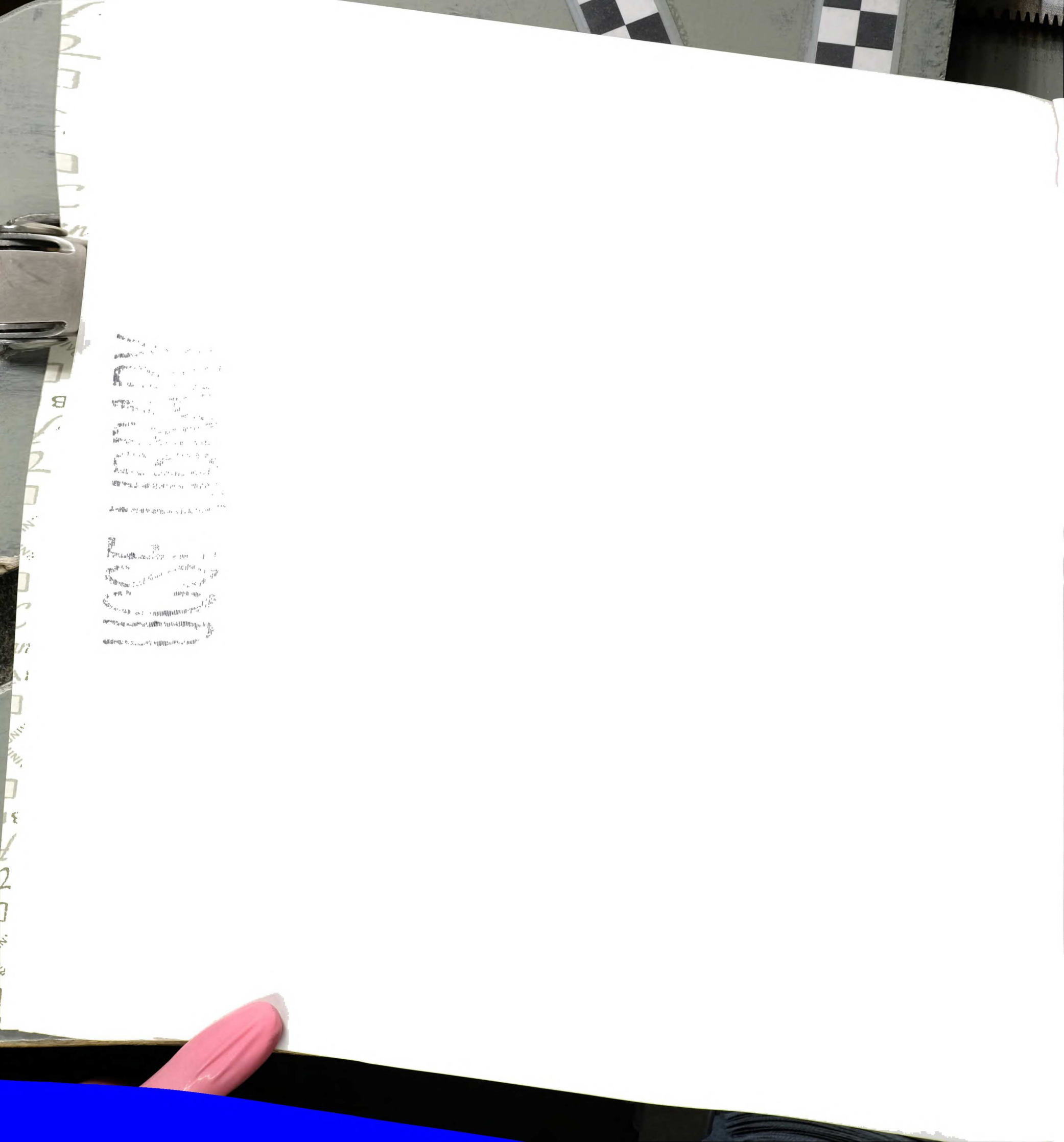


they are thought to be absent in Gram-positive bacteria like *M. smegmatis*. However, the unique cell wall of mycobacteria, with its outermost lipid bilayer, might have allowed mycobacteria to share the OMV pathway with Gram-negative bacteria. As there are numerous reports of *M. tuberculosis* lipid secretion both *in vitro* and *in vivo*, we propose that these lipids are packaged in these OMVs prior to secretion. Interestingly, the porin, MspA, is an abundant component of our secreted protein fractions. Naturally, we cannot directly link this released MspA to the OMVs, but it is tempting to speculate that MspA, which is largely buried in the outer lipid bilayer, is packaged into OMVs upon their secretion.

Since our discovery of these dramatic structures in *M. smegmatis*, other investigators have used a similar SEM approach to demonstrate that EFs and OMVs are conserved in *M. tuberculosis* (Dahl, 2005). Since both pathways are linked to virulence mechanisms in other organisms, future experiments must determine if they are similarly required for *M. tuberculosis* pathogenesis. Ultimately, the EFs and OMVs must be purified from both *M. smegmatis* and *M. tuberculosis*, and we must determine if, like the MmpL and Snm pathways, they function to deliver either lipid or protein virulence factors into the host during infection.

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1. The first part of the document is a list of names and addresses of the members of the committee. The names are listed in alphabetical order, and the addresses are given in full. The list is as follows:

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Mr. J. H. Smith, 123 Main St., New York, N. Y.
Mr. J. D. Jones, 456 Elm St., Boston, Mass.
Mr. W. E. Brown, 789 Oak St., Chicago, Ill.
Mr. R. L. Green, 101 Pine St., Philadelphia, Pa.
Mr. S. K. White, 202 Cedar St., St. Louis, Mo.
Mr. T. M. Black, 303 Maple St., Cincinnati, Ohio.
Mr. U. N. Gray, 404 Birch St., Portland, Me.
Mr. V. O. Hall, 505 Spruce St., San Francisco, Cal.
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1. The first part of the document is a list of names and addresses. The names are: John Doe, Jane Doe, and John Doe. The addresses are: 123 Main St, 456 Main St, and 789 Main St. The list is as follows:

Name	Address
John Doe	123 Main St
Jane Doe	456 Main St
John Doe	789 Main St

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The photograph shows a large, curved, segmented object, possibly a piece of machinery or a large pipe. It has various labels and markings, including the word "ELECTRIC" and some numbers. The object is curved in a semi-circle, and the segments are connected by joints. The background is dark and indistinct.

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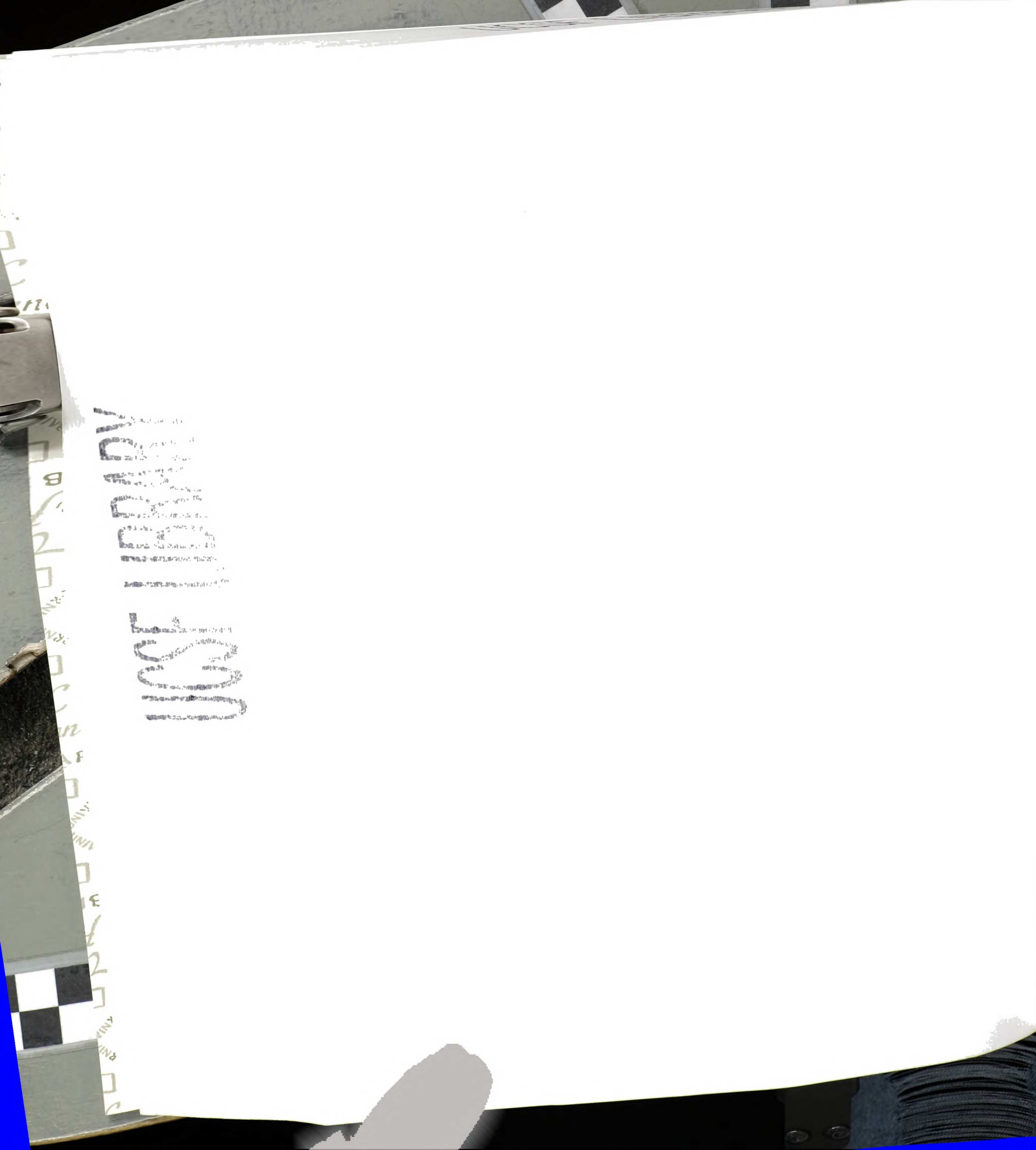
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