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

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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 nonpathogenic 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 multicomponent Snm secretory machine and to understand the role of this conserved system in mycobacterial biology.

Throughout evolution, numerous bacterial pathogens have acquired specialized protein secretion pathways to deliver effector proteins to host cells (3, 14, 34). 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 (14). 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 (36).

Initial clues of the existence of a Sec-independent protein secretion pathway in *Mycobacterium tuberculosis* came from studies of the immunodominant protein ESAT-6 (early secretory antigenic target, 6 kDa). This protein, along with its binding partner, CFP-10 (culture filtrate protein, 10 kDa), is efficiently secreted from *M. tuberculosis* cells but lacks N-terminal signal sequences that would target its secretion through the Sec pathway (28–30). The genes encoding ESAT-6 and CFP-10, *esxA* and *esxB*, are cotranscribed in a two-gene operon (1). 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 (4, 9, 19, 33). 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 *Mycobacterium bovis* during the repeated passage that led to the attenuated *M. bovis* 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 (23). 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 an alternative protein secretion pathway, termed the Snm (secretion in mycobacteria) pathway (10, 13, 31). 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 (31). 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 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 (10).

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 (17, 25, 31). 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) (31). These findings led to our working model that Snm2 recruits the ESAT-6–CFP-10 dimer to the cytoplasmic mem-

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brane 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 (12, 38), concerted ATP hydrolysis by Snm1 and Snm2 would promote ESAT-6–CFP-10 translocation through the secretory pore consisting of Snm4 (see Fig. 5).

Bioinformatic analysis of the *M. tuberculosis* genome has revealed several interesting features of the ESAT-6 and CFP-10 secretion pathway (4, 9, 19, 20, 33). 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 5 of the 11 loci, a conserved set of 10 genes, including *snm1-4* homologues, are syntenic with the *esx* operon. The proximity of the six other genes within these *esx* loci suggests that they encode 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 *Mycobacterium leprae*. Transposon mutagenesis in the ectotherm pathogen *Mycobacterium marinum* uncovered a homologous Snm pathway that was critical for host cell cytolysis and virulence (8). 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* (19). 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 5 of the 11 *esx* loci in *M. tuberculosis*, including the *esxA*/*esxB* locus and all of the surrounding known or putative *snm* genes (9) (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, nonpathogenic, rapid growth), we sought to utilize this organism 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 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.

MATERIALS AND METHODS

Protein preparation and analysis. *M. smegmatis* was inoculated from a single colony and grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.6 to 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 to 0.8), cells were washed and inoculated in 7H9 or Sauton's media without Tween 80 and grown to an OD_{600} of 0.8. Cells were harvested by centrifugation, and the cultured medium supernatant was filtered through a 0.22-μm-pore-size filter and concentrated with an Amicon Ultra-15 (5,000-molecular-weight cutoff; Millipore). For complementation experiments, medium was supplemented with kanamycin (20 μg/ml; Fisher Scientific). Whole-cell lysates and short-term culture filtrate (corresponding to 5 ml of original culture volume) fractions were precipitated with trichloroacetic acid and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 4 to 20% Tris-HCl Criterion gels (Bio-Rad). For immunoblotting of *M. smegmatis* SmESAT-6, polyclonal antibodies were raised against the N-terminal 20 amino acids (aa) plus a C-terminal cysteine (Covance Research Products, Inc.), and the antibody was affinity purified with the Sulfolink kit (Pierce Biotechnology). The antibodies against CFP-10 (K8493) and GroEL (HAT5) were kind gifts from P. Anderson. The antibody against KatG was a kind gift from 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 AflII 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 previously (11), and integrants were selected by growth 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 growth on 7H10 agar with 5% sucrose. Deletion generation was confirmed by PCR and Southern analysis. Strains are listed in Table 1, and more details of Southern analysis are given in Table 2.

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 (32). C-terminal 2× hemagglutinin (HA) (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.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)
Strains	
mc² 155	Wild-type
SCM17	Δ*Sm3866* (*snm5*)
SCM25	Δ*Sm3868*
SCM29	Δ*Sm3869* (*snm6*)
SCM30	Δ*snm1* (*Sm3870*)
SCM26	Δ*snm2* (*Sm3871*)
SCM27	Δ*esxB* (*cfp-10 Sm3874*)
SCM31	Δ*esxA* (*esat-6 Sm3875*)
SCM32	Δ*snm4* (*Sm3877*)
SCM28	Δ*Sm3882c* (*snm7*)
SCM21	Δ*mycP1* (*snm8 Sm3883c*)
SCM67	Wild-type + pMJ31
SCM72	Δ*Sm3866* + pMJ31
SCM41	Δ*Sm3866* + pSEC43 (*Sm3866-myc*)
SCM73	Δ*Sm3869* + pMJ31
SCM45	Δ*Sm3869* + pSEC47 (*Sm3869-myc*)
SCM79	Δ*Sm3882c* + pMJ31
SCM120	Δ*Sm3882c* + pSEC80 (*Sm3882c-2×HA*)
SCM80	Δ*mycP1* + pMJ31
SCM122	Δ*mycP1* + pSEC82 (*mycP1-2×HA*)
SCM76	Δ*esxB* + pMJ31
SCM141	Δ*esxB* + pSEC108 (*esxB*)
SCM77	Δ*esxA* + pMJ31
SCM142	Δ*esxA* + pSEC109 (*esxA*)
SCM143	Δ*esxA* + pMH406 (*M. tuberculosis esxA/B*)
Plasmids	
pMJ13	*groEL2* promoter, C-terminal in-frame 2× HA tag, Kan^r
pMJ31	*groEL2* promoter, C-terminal in-frame *myc* tag, Kan^r
pSEC43	*groEL2* promoter, *Sm3866-myc*, Kan^r
pSEC47	*groEL2* promoter, *Sm3869-myc*, Kan^r
pSEC108	*groEL2* promoter, untagged *esxB*, Kan^r
pSEC109	*groEL2* promoter, untagged *esxA*, Kan^r
pSEC72	*mycP1* promoter, C-terminal in-frame 2× HA tag, Kan^r
pSEC80	*mycP1* promoter, *Sm3882c-2×HA*, Kan^r
pSEC82	*mycP1* promoter, *mycP1-2×HA*, Kan^r
pMH406	*mop* promoter, *M. tuberculosis esxA/B*, Kan^r (see reference 10)

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

Strain	Enzyme(s)	Probe	Expected size of wild-type gene (bp)	Expected size of Δ gene (bp)	Wild-type gene product size (aa)	Δ gene product size (aa)
Δ*Sm3866* (*snm5*)	AflII, SphI	847-bp 3′ flank	4,076	2,374	294	31
Δ*Sm3868*	AflII, SphI	831-bp 5′ flank	4,076	2,311	574	38
Δ*Sm3869* (*snm6*)	AflII, BstBI	886-bp 5′ flank	17,751	5,618	479	29
Δ*snm1* (*Sm3870*)	AflII, NcoI	871-bp 3′ flank	3,822	1,546	742	32
Δ*snm2* (*Sm3871*)	AflII, SphI	841-bp 3′ flank	3,879	1,316	593	20
Δ*esxB* (*cfp-10*)	AflII, SphI	820-bp 3′ flank	4,619	3,741	100	13
Δ*esxA* (*esat-6*)	AflII, SphI	842-bp 3′ flank	4,619	3,419	95	9
Δ*snm4* (*Sm3877*)	AflII, SphI	855-bp 5′ flank	4,619	2,851	498	22
Δ*Sm3882c* (*snm7*)	AflII, NcoI	885-bp 3′ flank	7,552	1,758	464	16
Δ*mycP1* (*Sm3883c*)	PvuI	865-bp 5′ flank	2,294	1,695	449	15

To complement the *Sm3882c* and *mycP1* mutant secretion defect, the *groEL2* promoter in pMJ13 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 by using the QuikChange site-directed mutagenesis kit (Stratagene). The pMH406 *M. tuberculosis esxA*/*B* complementation plasmid was a kind gift from D. Sherman. Strains and plasmids are listed in Table 1.

RESULTS

Similarity of *esxA*/*esxB* loci in *M. smegmatis* and *M. tuberculosis*. The *M. smegmatis* genomic sequence was obtained from The Institute for Genomic Research (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* (Fig. 1A). The loci are organized in a similar fashion, and *M. smegmatis* possesses strong homologues for all of the known and putative *snm* genes (9). There are, however, some interesting differences between the two loci. For example, BLAST searches of a 4,459-bp region 5′ of the *Sm3881* gene revealed three genes that encode 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 snm3* homologue encodes 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* (9).

Analysis of SmESAT-6 and SmCFP-10 secretion under various growth conditions in *M. smegmatis*. To test our 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blotting with polyclonal antibodies raised against *M. smegmatis* SmESAT-6 and *M. tuberculosis* CFP-10. As shown in Fig. 1B, when cultured in 7H9 media, SmESAT-6 and SmCFP-10 are present in the cell-associated whole-cell lysate fraction but absent from the STCF fraction (lanes 1 and 2). Both proteins have apparent molecular masses similar to their homologues in *M. tuberculosis* (molecular mass of ESAT-6, 8.4 kDa; molecular mass of CFP-10, 11.5 kDa) (15, 27). 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.

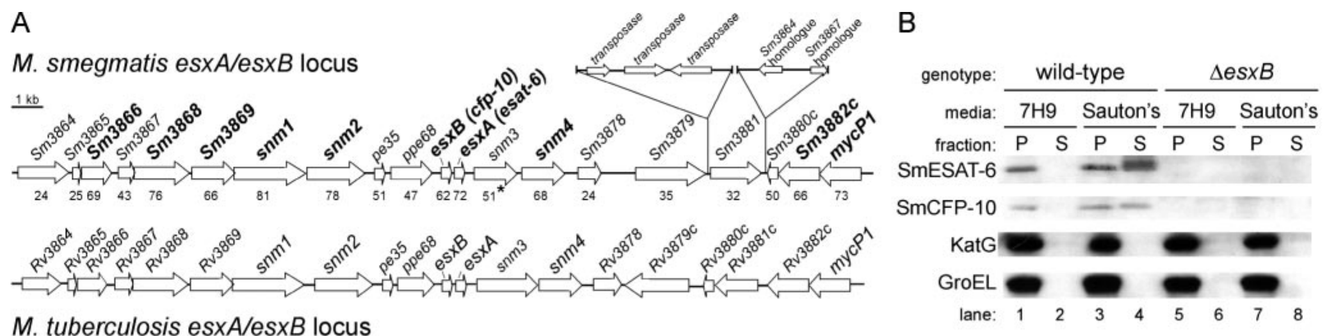

FIG. 1. *M. smegmatis* possesses a highly homologous *esxA*/*esxB* genomic locus and secretes SmESAT-6 and SmCFP-10 in a medium-dependent manner. (A) The *M. smegmatis* genomic sequence (http://www.tigr.org/tdb/ufmg/) was obtained from The Institute for Genomic Research, and the *M. tuberculosis* genomic sequence was obtained from Tuberculist (http://genolist.pasteur.fr/TubercuList/). The numbers below each open reading frame from *M. smegmatis* denote 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 gene names in boldface type represent those included in this study. The asterisks indicate that while the *snm3* homologue from *M. smegmatis* is 51% identical to its counterpart in *M. tuberculosis*, it lacks the 577-bp N-terminal portion found in the *M. tuberculosis* gene. (B) Wild-type or Δ*esxB* cells were grown to late log phase in 7H9 or Sauton's medium, fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blotting for the indicated proteins.

For technical reasons, STCF fractions are typically prepared from *M. tuberculosis* cultures grown in Sauton's medium, 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* (10, 30, 31), we also prepared STCF fractions from *M. smegmatis* cultured in Sauton's medium. Strikingly, both SmESAT-6 and SmCFP-10 are secreted into the STCF fraction when grown in this media (Fig. 1B, lanes 3 and 4). 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 (24, 37; data not shown), were exclusively cell associated at the time that all STCF fractions were harvested (Fig. 1B, lower panels). To show that our antibodies specifically recognized SmESAT-6 and SmCFP-10, we analyzed extracts prepared from a Δ*esxB* mutant (see below), as deletion of *esxB* in *M. tuberculosis* abolishes both SmESAT-6 and SmCFP-10 expression (31). As expected, the bands corresponding to SmESAT-6 and SmCFP-10 were missing in extracts from the *M. smegmatis* Δ*esxB* mutant (Fig. 1B, lanes 5 to 8). STCF protein profiles were qualitatively similar between 7H9 and Sauton's media (data not shown), suggesting that this medium 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 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 whether 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 the *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 two-step homologous recombination procedure, wild-type *M. smegmatis* was transformed with 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 (Fig. 2A; Table 1) (21). 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 obvious effect on bacterial growth, as the growth rate of each mutant strain was identical to that of the wild-type strain (data not shown). Accurate deletion of each gene was confirmed by Southern blot analysis, which revealed the expected patterns for the deletion strains (Fig. 2B; Table 2), 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

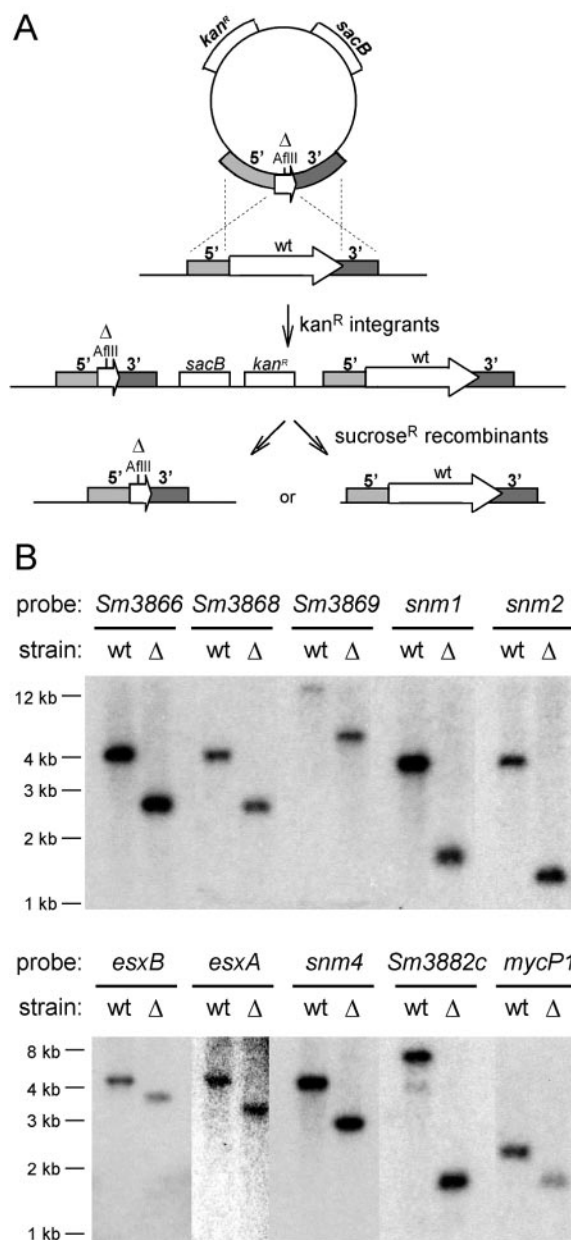

FIG. 2. In-frame deletion strategy and Southern blot analysis of mutants. (A) The in-frame deletion strategy involved PCR amplification of 800-bp 5′ and 3′ flanks to the wild-type (wt) gene of interest. The oligonucleotides were appropriately engineered to introduce a unique AflII restriction digest 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 counterselection 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 (Δ) strain revealed the expected bands. Genomic DNA was digested with AflII and one additional enzyme to confirm that the suicide plasmid had recombined in the proper location and that the AflII site and, thus, the correct reading frame were intact. For the *mycP1* Southern blot analysis, genomic DNA was digested with PvuI alone. More detail is given in Table 2.

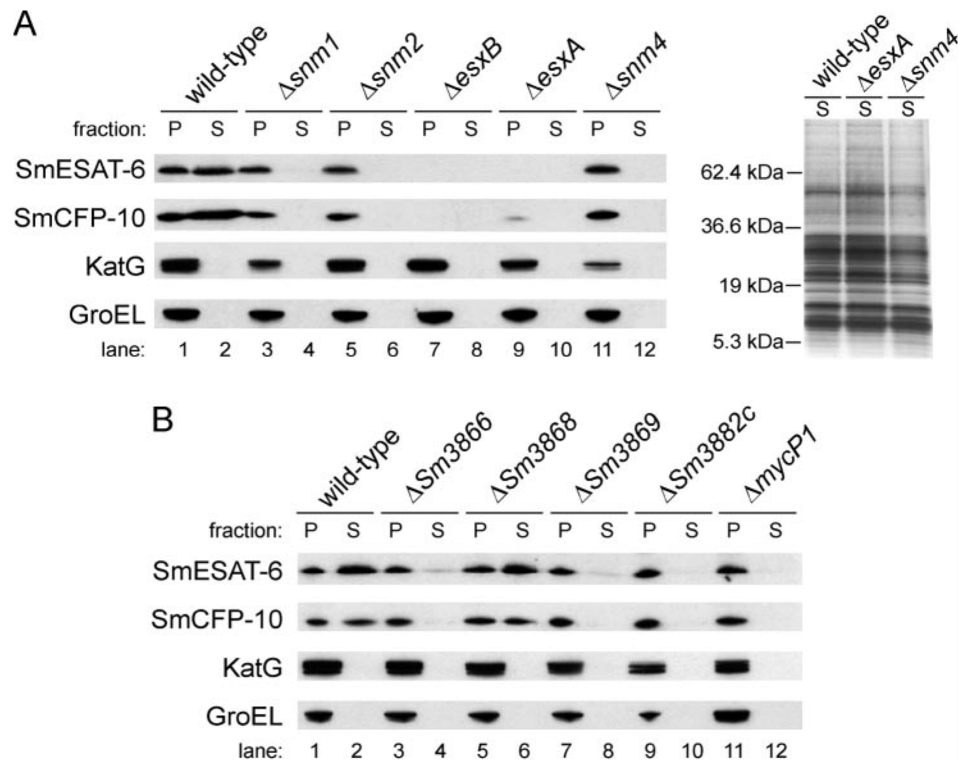

FIG. 3. *M. smegmatis* secretes SmESAT-6 and SmCFP-10 in an Snm-dependent manner, and four additional uncharacterized genes are also required for SmESAT-6 and SmCFP-10 secretion. (A and B) Wild-type and various mutant cells were grown to late log phase in Sauton's medium, fractionated into the cell-associated pellet (P) fraction and the short-term culture filtrate (S) fraction, and analyzed by Western blotting for the indicated proteins or by silver staining (right panel of panel A).

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 (Fig. 3A, lanes 1 to 6, 11, and 12). Furthermore, SmESAT-6 and SmCFP-10 are absent in their respective deletion strains (Fig. 3A, lanes 7 to 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 (31). 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 (Fig. 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 (Fig. 3A, right panel). In sum, these results clearly demonstrate that a functional Snm secretion apparatus exists in *M. smegmatis* and requires the components homologous 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 five putative *snm* genes from the *esxA*/*esxB* locus by using the same two-step strategy described above (Fig. 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 (Fig. 3B). Interestingly, *Sm3883c* encodes a previously identified membrane-anchored serine protease termed mycosin 1 (2, 6). Sm3866 shares limited homology with ABC ATPases involved in type I secretion (35), and *Sm3869* encodes a membrane protein with predicted ATP/GTP binding sites. Finally, *Sm3882c* is likely in an operon with *mycP1* and encodes 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 Snm pathway, and they suggest that a large, multicomponent secretion apparatus functions during SmESAT-6 and SmCFP-10 secretion in *M. smegmatis*.

Complementation of novel *snm* and *esx* mutants. 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 with 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 (Fig. 4A, lanes 1 to 10). Expression of Sm3882c and mycosin 1 from this promoter

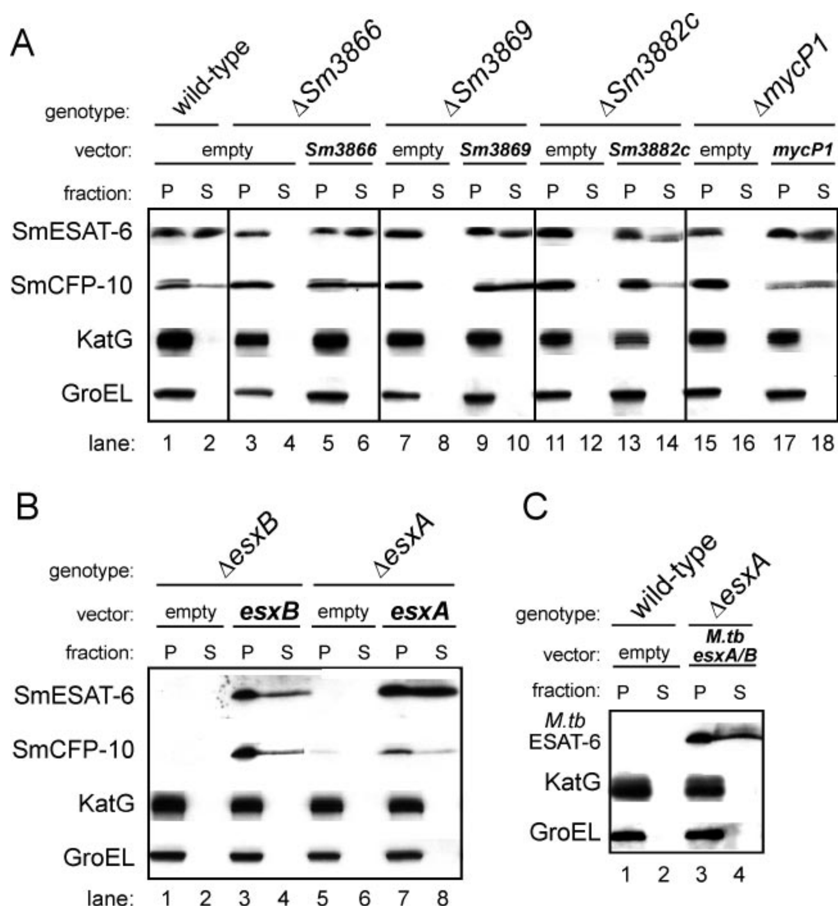

FIG. 4. Complementation of mutant strains with individual genes confirms that the Snm secretion pathway functions in *M. smegmatis*. (A and B) Various deletion strains were transformed with either an empty vector (pMJ13) or a multicopy episomal complementation plasmid constitutively expressing each individual gene C-terminally tagged with *myc*. In the case of *Sm3882c* and *mycP1* complementation, the complementation plasmid expressed the individual genes tagged with 2× HA from the presumed endogenous promoter (505-bp fragment upstream of *mycP1*). (C) 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 (10; data not shown). All cells were cultured in Sauton's medium with kanamycin, fractionated, and analyzed by Western blotting.

caused cell toxicity (data not shown). Therefore, as both genes are likely in an operon (Fig. 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 Fig. 4B, lanes 11 to 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 (Fig. 4, 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 Δ*esxA* and Δ*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 strains rescued the synthesis and secretion of both SmCFP-10 and SmESAT-6 compared to vector-transformed knockout strains (Fig. 4B, lanes 1 to 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 whether the *M. tuberculosis* ESAT-6 and CFP-10 homologues could be secreted by the *M. smegmatis* system (Fig. 4C). Importantly, the *M. tuberculosis* ESAT-6 monoclonal antibody, which readily detects secreted *M. tuberculosis* ESAT-6 (31), does not recognize endogenous *M. smegmatis* SmESAT-6 (lanes 1 and 2). Introduction of an episomal plasmid, in which expression of *M. tuberculosis esxA* and *esxB* is driven by the synthetic *mop* promoter (10), into Δ*esxA* cells resulted in synthesis and secretion of the *M. tuberculosis* homologues (lanes 3 and 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

In this study, we have presented the first functional characterization of the Snm alternative protein secretion pathway in

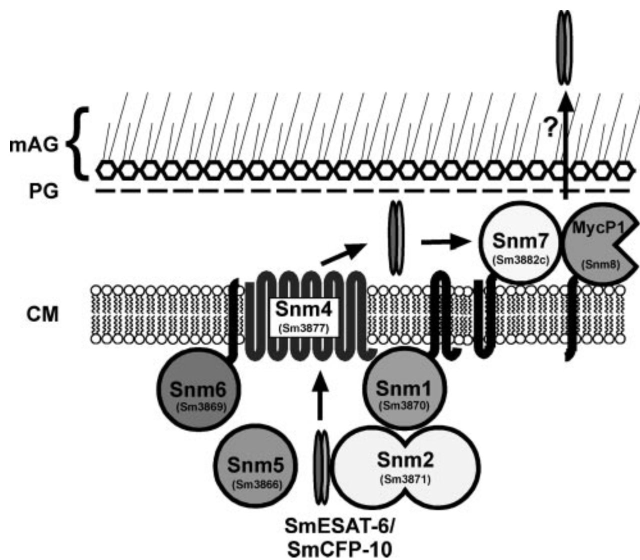

FIG. 5. Model of the Snm secretion pathway in *M. smegmatis.* See Discussion for details of the model. CM, cytoplasmic membrane; PG, peptidoglycan layer; mAG, mycolyl arabinogalactan layer. Transmembrane domains were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and topology is inferred based on predicted function and some experimental evidence (6).

a nonpathogenic bacterium. *M. smegmatis* possesses an *esx/snm* locus homologous to that of *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 (Fig. 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 (18). 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 (2, 6). Given the functional conservation between the Snm pathway in *M. smegmatis* and *M. tuberculosis*, it is likely that *mycP1* and *snm5-7* 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 medium but not in 7H9. Because there are numerous differences between Sauton's and 7H9 medium, it seems likely that the Snm system is responsive to one or more components of the growth medium. Our efforts to determine the precise component responsible for this regulation have been unsuccessful. For example, while Sauton's medium, compared to 7H9, has no dextrose, calcium, or zinc, supplementing Sauton's medium with these components did not suppress SmESAT-6 and SmCFP-10 secretion (data not shown). 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 bacteria, which also require strict medium conditions for active protein export (5, 26).

The unique mycobacterial cell wall represents an enormous barrier to protein secretion, and how ESAT-6 and CFP-10 are translocated across all of the layers of the thick mycobacterial cell wall remains unknown. In addition to the peptidoglycan layer, mycobacteria possess hydrophobic long-chain mycolic acids esterified to a polymeric arabinogalactan layer (Fig. 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 (16, 13, 22). 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 may 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. (7) 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. 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 of *M. smegmatis* will not only help delineate the mechanism of Snm secretion but perhaps uncover the evolutionarily conserved role of the Snm pathway.

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