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Protease domain and transmembrane domain of the type VII secretion mycosin protease determine system-specific functioning in mycobacteria

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Mycobacteria use type VII secretion systems to secrete proteins across their highly hydrophobic diderm cell envelope. Pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium marinum,* have up to five of these systems, named ESX-1 to ESX-5. Most of these systems contain a set of five conserved membrane components, of which the four Ecc proteins form the core membrane-embedded secretion complex. The fifth conserved membrane protein, mycosin protease (MycP), is not part of the core complex but is essential for secretion, as it stabilizes this membrane complex. Here we investigated which MycP domains are required for this stabilization by producing hybrid constructs between $MycP_1$ and $MycP_5$ in *M. marinum* and analyzed their effect on ESX-1 and ESX-5 secretion. We found that both the protease and transmembrane domain are required for the ESX system-specific function of mycosins. In addition, we observed that the transmembrane domain strongly affects MycP protein levels. We also show that the extended loops 1 and 2 in the protease domain are probably primarily involved in MycP stability, whereas loop 3 and the $MycP_5$-specific loop 5 are dispensable. The atypical propeptide, or N-terminal extension, is required only for MycP stability. Finally, we show that the protease domain of $MycP_{P1}$, encoded by the *esx-P1* locus on the pRAW plasmid, is functionally redundant to the protease domain of $MycP_5$. These results provide the first insight into the regions of mycosins involved in interaction with and stabilization of their respective ESX complexes.

Mycobacteria, including *Mycobacterium tuberculosis*, have a set of specialized secretion systems, called type VII secretion (T7S)[2] systems, to secrete proteins across their highly hydrophobic cell envelope (1). This group of bacteria contains up to five genomic clusters coding for these T7S systems, named ESX-1 to ESX-5 (1, 2). Three of these systems, ESX-1, ESX-3, and ESX-5, have been shown to be functional and to play critical roles in virulence and bacterial physiology (3–6). The ESX-1 system is required for phagosomal rupture both in *M. tuberculosis* and *Mycobacterium marinum* (3, 7, 8), and is therefore a crucial factor for completing the intraphagocytic stage of these pathogens. The importance of ESX-1 for virulence is further illustrated by the absence of the *esx-1* region in the live vaccine strain *Mycobacterium bovis* BCG (4). The ESX-3 and ESX-5 systems have very different functions, as they have been linked to iron and fatty acid uptake, respectively (5, 6, 9). Because of their role in metabolite and nutrient uptake, both of these systems are essential for *in vitro* growth in pathogenic species. However, the essentiality of the ESX-5 system can be circumvented in *M. marinum* by permeabilizing the unique mycobacterial outer membrane, either by mutating genes involved in the biosynthesis of the outer membrane lipid phthiocerol dimycocerosate or by the introduction of MspA, an outer membrane porin from the nonpathogenic *Mycobacterium smegmatis* (6).

In the last decade, information has been obtained about the composition of the membrane-embedded ESX complexes (10, 11) and the structure and (possible) functioning of several of its components, *e.g.* of EccB, EccC, and EccD (12, 13). This has recently seen a strong leap forward with the first structure of the core ESX-5 secretion complex of *Mycobacterium xenopi*, imaged by negative-stain EM (14). From the observed 6-fold symmetry together with stoichiometry measurements, it can be deduced that the ESX-5 complex consists of six copies of each of the four conserved membrane components that make up the complex, *i.e.* $EccB_5$, $EccC_5$, $EccD_5$, and $EccE_5$ (14). The data on the *M. xenopi* ESX-5 complex match the composition determined previously for the ESX-1 and ESX-5 systems of *M. marinum* and the ESX-5 system of *M. bovis* BCG by protein pull-down experiments (10, 11). Notably, the fifth membrane component, the mycosin protease (MycP), was not detected in significant amounts in any of these purified complexes. Despite this, mycosins are essential for the proper functioning of the ESX-1 and ESX-5 systems, as $mycP_1$ or $mycP_5$ knockouts result in a secretion deficiency by the respective systems in different species (11, 15), and transposon mutagenesis experiments indicate that $MycP_3$ is also essential in *M. tuberculosis* (16, 17). Furthermore, mycosins are widely conserved in T7S, not only in all mycobacterial ESX systems but also in T7S clusters of other

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Actinobacteria, suggesting that they have a critical function in T7S (18, 19). Recently, we showed that both $MycP_1$ and $MycP_5$ stabilize their respective ESX complexes in *M. marinum*, explaining their essentiality for T7S in mycobacteria (11).

Mycosins belong to the family of subtilisin-like proteases, and the available crystal structures indeed show a subtilisin-like fold (20, 21). However, there are also some differences from classical subtilisins, most prominent for the so-called propeptide. Although subtilisins contain an N-terminal propeptide that blocks the active site, which is cleaved off upon protein maturation to activate the protease, for mycosins, this N-terminal extension is not processed but remains part of the mature protease domain. Furthermore, this extension is not blocking the substrate binding pocket but is elongated and tightly wrapped around the protease domain (20–22). As the N-terminal region of mycosins does not contain any of the typical characteristics of a propeptide (22), we refer to it as the N-terminal extension (NE) of mycosins in this study. Compared with typical subtilisins, the mycosins have a deeper active-site groove because of the presence of the N-terminal extension in the mature protein and three extended loops that surround the active site (20, 21). This suggests that mycosins have a higher substrate selectivity compared with most subtilisin-like proteases. Finally, mycosins also have a C-terminal transmembrane (TM) domain, connected to the protease domain by an ~28-amino-acid-long linker region. Because of the presence of a typical signal sequence preceding the N-terminal extension and a positively charged short C-terminal tail, the protease domain is predicted to be localized in the periplasm.

So far, the number of identified substrates of mycosins is extremely limited. Only $MycP_1$ has been shown to cleave the C terminus of EspB, one of the substrates of the ESX-1 system, in *M. tuberculosis* and *M. marinum* (11, 15). Surprisingly, the proteolytic activity of $MycP_1$ is not required for the essential function of this protein in secretion (11, 15). Also, an active-site mutant of $mycP_5$ can still mediate ESX-5 secretion in *M. marinum* (11). This discrepancy suggests that the second essential function of mycosins is not dependent on their proteolytic activity. As already mentioned, mycosins are involved in stabilization of their respective ESX complex without having a role in complex assembly (11). This conclusion is based on the observation that we could detect the ESX-1 or ESX-5 complex in a $mycP_1$ or $mycP_5$ knockout, respectively, when we cross-linked cell envelope fractions prior to detergent extraction of the membrane-embedded ESX complexes (11). Therefore, the ESX complex is apparently formed in the absence of mycosin but dissociates rapidly upon detergent extraction. The proteolytic activity of MycP is not required for this function, as an active site mutation did not affect the stabilizing effect of $MycP_1$ or $MycP_5$ on its respective ESX complex (11). However, the MycP domain responsible for this function has yet to be identified.

Here we set out to identify the domains of $MycP_1$ and $MycP_5$ that are involved in the essential role of mycosins in T7S in *M. marinum* by producing specific truncated versions and hybrid proteins in which individual domains of $MycP_1$ and $MycP_5$ were exchanged. Our results show that the N-terminal extension is required for structural integrity, whereas both the protease and TM domains of $MycP_1$ and $MycP_5$ are system-specific and essential for the functionality of their respective ESX complexes. By further dissecting the protease domain, we found that the primary function of the extended loops is not related to the stabilization of the membrane complex. Finally, we show that the protease domain of $MycP_{P1}$ of the plasmid-encoded ESX-P1 system can complement the function of the protease domain of $MycP_5$. This study provides important functional insights into the different domains of the mycosin proteases.

Results

The N-terminal extension and the TM domain are essential for mycosin functioning

To determine which domains are involved in the essential function of mycosins in T7S, we produced $mycP_1$ and $mycP_5$ truncations in which either the NE or the TM domain was removed (Figs. 1A and 2A) and analyzed their functionality in the previously described $mycP_1$ and of $mycP_5$ knockout strains of *M. marinum* (11). To be able to dissect the functionality of the different $MycP_5$ constructs in ESX-5 secretion, the essentiality of ESX-5 for *in vitro* growth was alleviated by introduction of the outer membrane porin MspA (6, 23). As reported previously (11, 15), knockout of both $mycP_1$ and $mycP_5$ resulted in complete secretion deficiency by their respective ESX systems. We showed this deficiency by the absence of the ESX-5 substrate group of PE_polymorphic GC-rich repetitive sequences (PGRS) proteins in the pellet and supernatant for the $mycP_5$ knockout (Fig. 1C, lanes 2 and 12) and the absence of the ESX-1 substrates EsxA and EspB in the supernatant fraction of the $mycP_1$ knockout (Fig. 2C, lane 12). The secretion by the mutants can be complemented by exogenous expression of their respective WT copies bearing a C-terminal HA tag for detection (Figs. 1C, lane 13, P5, and 2C, lane 13, P1). As expected, secretion could not be restored by exogenous expression of $MycP_1$ in the $mycP_5$ knockout strain and vice versa (Figs. 1C, lane 14, and 2C, lane 14). An additional observation was that, although $MycP_5$ is larger than $MycP_1$, the HA signal for $MycP_5$ showed a lower molecular weight compared with $MycP_1$ (Fig. 1B, lanes 3 and 4, P1 and P5). Based on the size of the detected fragment (~37 kDa), we concluded that $MycP_5$ is processed somewhere in its protease domain, which we will discuss further below.

Next we determined the effect of deleting the N-terminal extension of $MycP_5$ (P5ΔNE) and $MycP_1$ (P1ΔNE). We found that, for both $MycP_5$ and $MycP_1$, this deletion resulted in complete loss of protein secretion by their respective ESX systems (Figs. 1C, lane 15, and 2C, lane 15). In addition, although we could readily detect HA-tagged WT $MycP_5$ and $MycP_1$ in bacterial pellet fractions, we were unable to detect these truncated proteins via the same C-terminal HA tag in the same bacterial fraction (Figs. 1B, lane 5, and 2B, lane 5), suggesting that the N-terminal extension of mycosins is required for protein stability.

Although deletion of the NE reduced the stability of mycosins, removing the C-terminal TM domain resulted in a strong increase in mycosin levels compared with their WT versions. Although this effect was observed for both $MycP_5$ and $MycP_1$, especially in the case of $MycP_1ΔTM$, the increase in HA signal was of such intensity that the other hybrid constructs were not or barely detectable without overexposing the $MycP_1ΔTM$ sig-
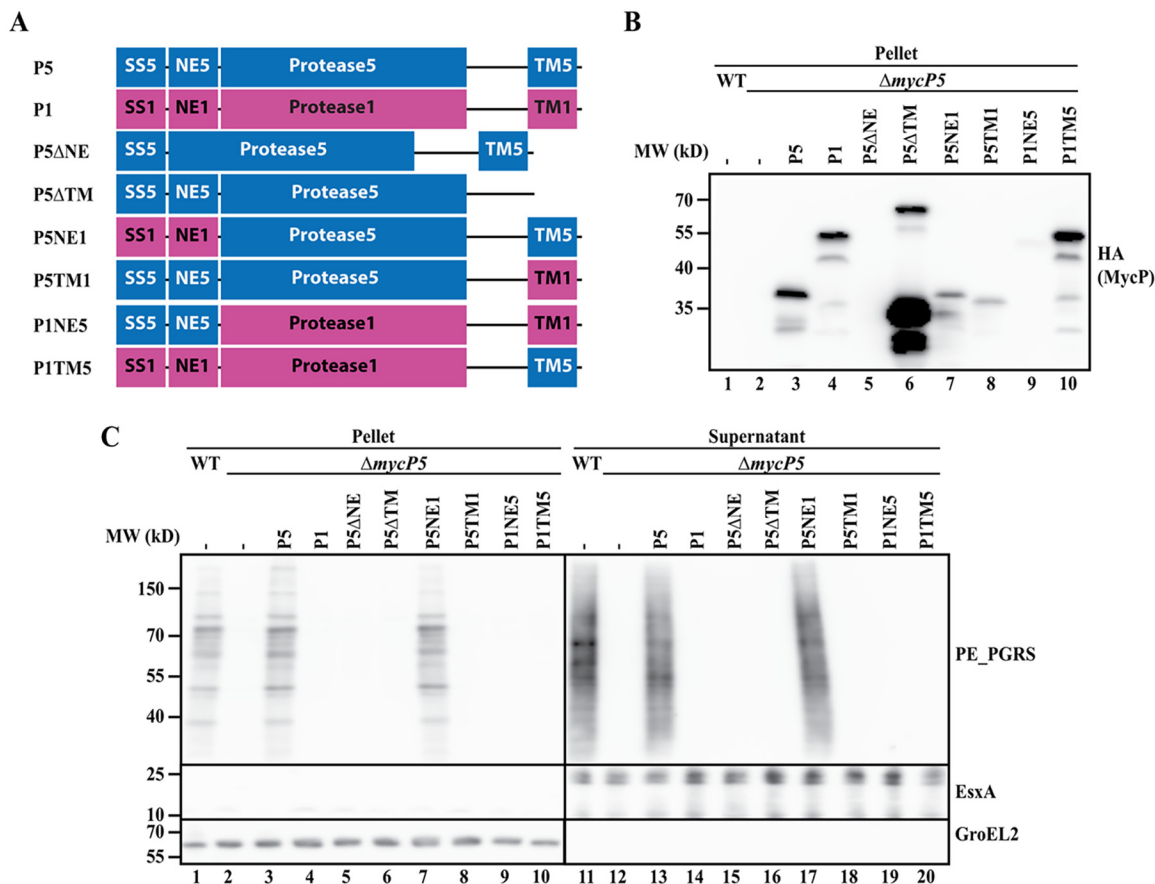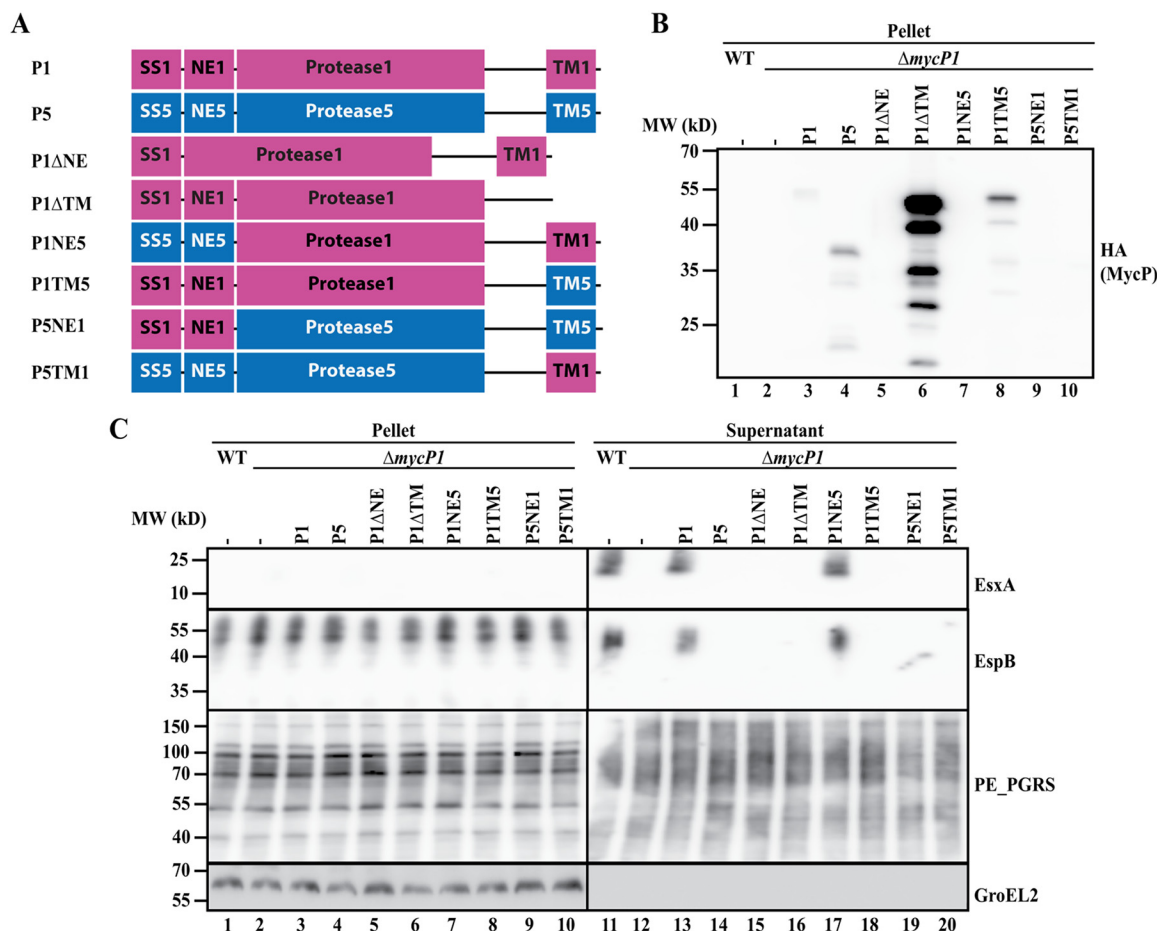
Figure 1. *A*, schematic overview of (hybrid or truncated) *mycP₅* (*blue*) and *mycP₁* (*purple*) constructs used to complement a *mycP₅* knockout in *M. marinum*. The constructs contain the following parts: *SS*, signal sequence; *NE*, N-terminal extension; *Protease*, protease domain (including the linker region between the protease and TM domain); *TM*, transmembrane domain (including the short C-terminal tail). The numbers indicate the origin of the fragment, *i.e. 1* for MycP₁ and *5* for MycP₅. The exact residues of the swapped domains are indicated in Fig. S4. In addition, all proteins have a C-terminal HA tag. *B*, immunoblot detection of the HA-tagged constructs expressed in the *mycP₅* knockout. *MW*, molecular weight. *C*, immunoblot analysis of cell pellets and supernatants of WT *M. marinum* and a *mycP₅* knockout, complemented with the constructs depicted in *A*. Proteins were visualized with anti-PE_PGRS (ESX-5 substrates), anti-EsxA (ESX-1 substrate, supernatant loading control), and anti-GroEL2 (cytosolic loading control).

nal (Figs. 1*B*, *lane 6*, *P5ΔTM*, and 2*B*, *lane 6*, *P1ΔTM*). Therefore, we included an additional immunoblot lacking the truncated MycP₁ construct to allow more accurate comparison with WT MycP₁ or MycP₅ (Fig. S1). Interestingly, for the MycP₅ truncate, besides the C-terminal portion of ~37 kDa, an ~65 kDa band could be detected, which probably represented the unprocessed full-length protein. However, although present at high levels, the myosin constructs lacking the TM domain were unable to complement ESX secretion of their respective knockouts (Figs. 1*C*, *lane 16*, and 2*C*, *lane 16*). Furthermore, expressing the highly stable truncated MycP₅ΔTM did not result in a dominant-negative effect on ESX-5–mediated secretion in WT bacteria (Fig. S2). These results show that both the N-terminal extension and the C-terminal TM domain are required for the essential function of myosins within their respective ESX systems.

Both the protease and TM domain of MycP₁ and MycP₅ are ESX system–specific

To distinguish between a general role in protein stability (for the N-terminal extension) or subcellular localization (for the TM domain) and a system-specific function of the separate domains, we created hybrid *mycP₁* and *mycP₅* constructs in which individual domains were exchanged between the two

myosins, again with a C-terminal HA tag for detection (Figs. 1*A* and 2*A*). As deletion of the N-terminal extension resulted in a strong decrease in mycosin levels, we first wanted to confirm whether this domain is only involved in structural stability and does not play a system-specific role. Indeed, although we did observe a decrease in mycosin levels when we exchanged this domain between MycP₅ and MycP₁ (Figs. 1, *A and B*, *lane 7*, *P5NE1* and 2, *A and B*, *lane 7*, *P1NE5*, and Fig. S1, *lane 5*), both constructs were still able to complement ESX-5 or ESX-1 secretion of the *mycP₅* and *mycP₁* knockouts, respectively (Figs. 1*C*, *lane 17*, and 2*C*, *lane 17*). With a similar approach, we exchanged the C-terminal TM domain of the mycosins to investigate whether they have a system-specific role. Although we observed decreased expression of MycP₅ with the TM domain of MycP₁ (Fig. 1*B*, *lane 8*, *P5TM1*), the opposite MycP₁ construct showed substantial expression levels (Fig. 2*B*, *lane 8*, *P1TM5*, and Fig. S1, *lane 6*). Both constructs were unable to complement secretion by *mycP₅* or *mycP₁* knockout (Figs. 1*C*, *lane 18*, and 2*C*, *lane 18*), strongly suggesting that the TM domain of mycosins does not only function as a membrane anchor but that it has a specific function for its ESX system. Interestingly, we also tested whether all constructs were able to alleviate the essentiality of *mycP₅*, *i.e.* by mediating growth in the absence of the

Figure 2. *A*, schematic overview of (hybrid or truncated) *mycP₁* (*purple*) and *mycP₅* (*blue*) constructs used to complement a *mycP₁* knockout in *M. marinum*. All constructs contain a C-terminal HA tag. See Fig. 1A for an explanation of the abbreviated domains. *B*, immunoblot detection of the HA-tagged constructs, expressed in the *mycP₁* knockout. *MW*, molecular weight. *C*, immunoblot analysis of cell pellets and supernatants of WT *M. marinum* and a *mycP₅* knockout, complemented with the constructs depicted in *A*. Proteins were visualized with anti-EsxA and anti-EspB (ESX-1 substrates), anti-PE_PGRS (ESX-5 substrates, supernatant loading control), and anti-GroEL2 (cytosolic loading control).

outer membrane porin MspA (Table S2). All constructs that could complement protein secretion also supported growth of the *mycP₅* mutant in the absence of MspA. Although P5TM1 was unable to restore ESX-5 secretion in the *mycP₅* knockout, this construct did allow this mutant strain to grow, albeit with a strongly reduced growth rate. This suggests that this hybrid construct is able to complement the *mycP₅* mutant to some extent.

Finally, we determined whether the protease domain is also involved in the essential function of mycosins by introducing the hybrid constructs in the opposite *mycP* knockout, *e.g.* P1NE5 and P1TM5 in the *mycP₅* knockout and vice versa (Figs. 1A and 2A). These constructs were also unable to complement the mycosin knockouts and were thus unable to restore secretion of the analyzed ESX systems (Figs. 1C, *lanes 19* and *20*, and *2C*, *lanes 19* and *20*). As we already showed that exchanging the N-terminal extension has no effect on the functionality of MycP₁ or MycP₅, the inability of P1TM5 and P5TM1 to complement the *mycP₅* and *mycP₁* knockout, respectively, must be contributed to the exchanged protease domain. In conclusion, although the N-terminal extension has a system-specific function in protein stability, both the protease and C-terminal TM domain of mycosins are required and specific for essential function within their respective ESX systems.

The extended loops of the protease domain are either dispensable or involved in structural stability

Next we set out to identify specific regions within the protease domain that are involved in the essential function of mycosins. A distinctive feature of mycosins compared with classical subtilisins is three extended loops that are present in the protease domain (loops 1–3) (Fig. 3A). In addition to these three conserved loops, MycP₅ has an additional large extension to which we will refer as loop 5 in this study. This loop is not present in MycP₁. In a structural model of MycP₅, produced from the available MycP₁ structure from *M. smegmatis* (20), loop 5 remained unstructured, and we therefore only show the start and end of it in the model shown in Fig. 3A. Because loops 1–3 and loop 5 are unique for mycosins, we hypothesized that they could be involved in the essential function of mycosins. By individually deleting these four loops of MycP₅, we investigated their effect on stability and/or functionality. Deleting loop 1 or 2 resulted in reduced stability of MycP₅; especially loop 2 deletion showed almost complete loss of MycP₅ stability (Fig. 3B). In addition, both constructs resulted in complete loss of ESX-5 secretion (Fig. 3C, *lanes 13* and *14*). In contrast, deleting loop 3 or loop 5 had little to no effect on MycP₅ stability or on ESX-5
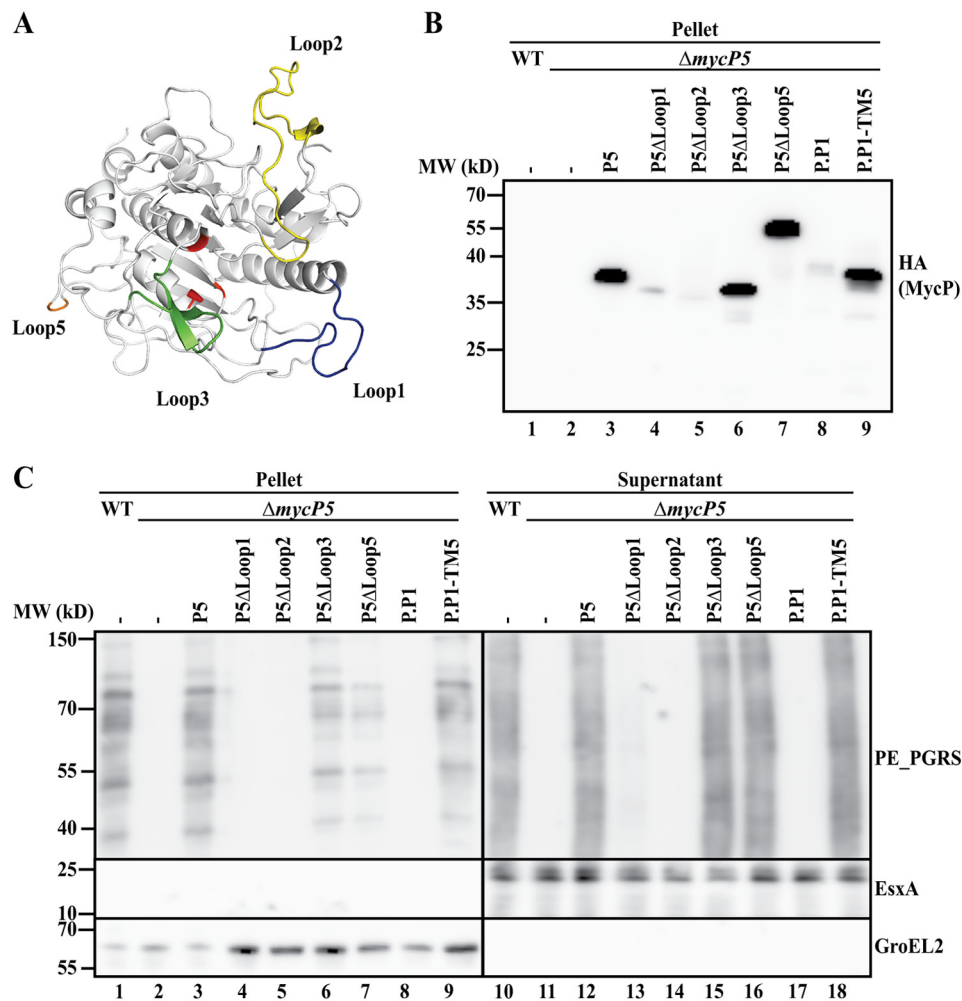
Figure 3. *A*, model of MycP$_5$, based on the available crystal structure of MycP$_1$. The catalytic triad of the protease is depicted in *red*, and the three extended loops (1–3) surrounding the catalytic groove are depicted in *blue*, *yellow*, and *green*, respectively. The two residues bordering the extended loop 5 are depicted in *orange*, whereas loop 5 itself is not shown in this model. *B*, immunoblot detection of HA-tagged constructs expressed in the *mycP$_5$* knockout. *P5*, WT *mycP$_5$*; *P5ΔLoop1/2/3/5*, *mycP$_5$* without loop 1, 2, 3, or 5; *P.P1*, *mycP$_{P1}$*; *P.P1-TM5*, hybrid *mycP$_{P1}$* with the TM region of *mycP$_5$*. The exact residues of the deleted and swapped regions are indicated in Fig. S4. *MW*, molecular weight. *C*, immunoblot analysis of cell pellets and supernatants of WT *M. marinum* and a *mycP$_5$* knockout, complemented with (truncated) *mycP$_5$* constructs or (hybrid) *mycP$_{P1}$* with the TM region of *mycP$_5$* (see Fig. S4 for the swapped residues). Proteins were visualized with anti-PE_PGRS (ESX-5 substrates), anti-EsxA (ESX-1 substrate, supernatant loading control), and anti-GroEL2 (cytosolic loading control).

secretion (Fig. 3, *B*, *lanes 6* and *7*, and *C*, *lanes 15* and *16*). Interestingly, deleting loop 5 resulted in a higher detected molecular weight compared with the WT HA-tagged MycP$_5$ (Fig. 3*B*, *lanes 3* and *7*), indicating that MycP$_5$ is cleaved somewhere in this loop and that this processing is not required for proper functioning. To investigate whether this processing is performed by MycP$_5$ or by a different component of the ESX-5 system, we introduced both a WT and an active-site mutant variant of HA-tagged MycP$_5$ in *M. smegmatis*, which lacks the ESX-5 system. However, we found that, in both cases, MycP$_5$ was present as the processed form, showing that the responsible protease is unrelated to the ESX-5 system (Fig. S3*C*).

Subsequently, we analyzed the role of loop 1 and 2 in protease activity using the ability of purified MycP$_1$ to process its substrate EspB *in vitro*. Although we were unable to produce stable MycP$_1$ with the deletion of loop 2, deletion of loop 1 resulted in substantial expression of the MycP$_1$ protease domain. This purified protein was also active, as we could not detect a differ-

ence in the activity of purified WT MycP$_1$ compared with the loop 1 truncation; in both cases, EspB was successfully processed *in vitro* (Fig. S3*D*, *lanes 2* and *3*, *P1* and *P1ΔL1*). Finally, we exchanged the loop 1 of full-length MycP$_5$ with the loop of MycP$_1$ and analyzed its expression and functionality in the *mycP$_5$* knockout strain. This hybrid protein was stable (Fig. S3*A*, *lane 4*, *P5-L1.P1*) and resulted in successful complementation of ESX-5 secretion (Fig. S3*B*, *lane 8*). Together, the results show that, although loop 3 and loop 5 are dispensable for MycP$_5$ stability and its essential role in ESX-5 functioning, loop 1 and 2 seem to have a structural role, similar to the N-terminal extension.

The protease domain of MycP$_5$ can be functionally substituted by the protease domain of MycP$_{P1}$ of the plasmid-encoded ESX-P1 system

As we were unable to obtain further functional insight from analysis of the extended loops in MycP$_5$, and as there are no other distinctive regions or structures within the protease domain, we looked for a different approach. More recently,
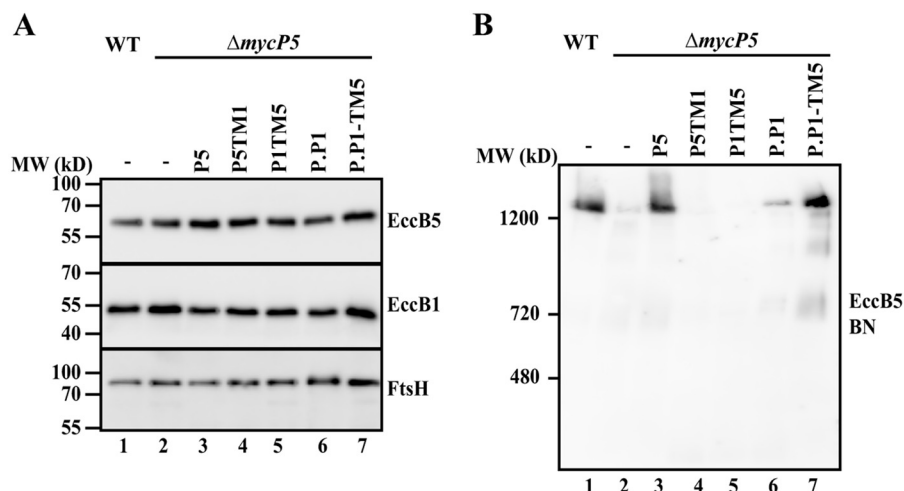
Figure 4. *A*, immunoblot analysis of solubilized cell envelope fractions of WT *M. marinum* and a *mycP5* knockout, complemented with WT *mycP5* (*P5*), hybrid constructs of *mycP5* with the TM region of *mycP1* (*P5TM1*) or *mycP1* with the TM region of *mycP5* (*P1TM5*), WT *mycP$_{P1}$* (*P.P1*), and a hybrid construct of *mycP$_{P1}$* with the TM region of *mycP5* (*P.P1-TM5*). Proteins were visualized with anti-EccB$_5$ and, as loading controls, anti-EccB$_1$ and anti-FtsH. *B*, blue native PAGE immunoblot analysis of solubilized cell envelope fractions; the ESX-5 complex was visualized with anti-EccB5.

plasmid-localized *esx* clusters have been described for mycobacteria, most notably the *esx-P1* cluster localized on the pRAW plasmid found in *M. marinum* E11 (24), involved in bacterial conjugation. Interestingly, the ESX-P1 system is most closely related to the ESX-5 system, *e.g.* MycP$_{P1}$ has ~59.5% identity to MycP$_5$ (Fig. S4 and Table S1), which is substantially higher than the identity of MycP$_5$ to MycP$_1$ (~46%, Table S1). Because of this high sequence similarity, we set out to investigate to what extent *mycP$_{P1}$* is able to complement the *mycP$_5$* knockout of *M. marinum* M, which does not contain the *esx-P1* cluster. Unfortunately, we were able to detect only low protein levels of the HA-tagged MycP$_{P1}$ (Fig. 3*B*, *lane 8, P.P1*), and this construct did not result in successful complementation of the *mycP$_5$* knockout (Fig. 3*C*, *lane 17*). In addition to this, the HA signal of the MycP$_{P1}$ protein showed a comparable size as that of MycP$_5$ (Fig. 3*B*, *lane 3*), indicating that MycP$_{P1}$ is also processed, which is in line with the observation that this protein contains a loop 5 extension similar to MycP$_5$ (Fig. S4). As we already determined that the TM domain plays an important role, we exchanged the TM domain of MycP$_{P1}$ with that of MycP$_5$. Interestingly, this not only resulted in a substantially more stable protein (Fig. 3*B*, *lane 9, P.P1-TM5*), but it was also capable of restoring ESX-5 secretion in the *mycP$_5$* knockout (Fig. 3*C*, *lane 18*). These results show that the protease domain of MycP$_{P1}$ has functional similarity to that of MycP$_5$ and further strengthens the notion that the TM domain of mycosins has a highly system-specific function.

The protease and TM domain of MycP are both involved in stabilization of the ESX complex

To further confirm that the essential role of the protease and TM domain is linked to the previously determined crucial role of mycosins in the stabilization of their respective ESX complex (11), we investigated the effect of expressing our hybrid constructs in the *mycP$_5$* knockout on the ESX-5 membrane complex. First, we established that there were no effects of our introduced constructs on the production or stability of EccB$_5$, one of the components of the ESX-5 membrane complex (Fig.

4*A*). Next we investigated the stability of the ESX-5 membrane complex. As shown previously (11), the 1.8-MDa ESX-5 complex could be solubilized in a stable form from the WT strain and visualized by blue native PAGE, whereas solubilized complex levels were strongly reduced in the *mycP$_5$* knockout (Fig. 4*B*, *lanes 1* and *2*). Exchanging either the TM or protease domain of MycP$_5$ with that of MycP$_1$ (P5TM1 and P1TM5) did not restore stability of the ESX-5 complex in the *mycP$_5$* knockout (Fig. 4*B*, *lanes 4* and *5*), which was expected, as these constructs were also unable to successfully restore secretion (Fig. 1*C*). In contrast to this, although MycP$_{P1}$ was unable to complement secretion, we were able to detect a slight increase in ESX-5 complex levels when we introduced MycP$_{P1}$ (Fig. 4*B*, *lane 6, P.P1*). Complementation of the hybrid MycP$_{P1}$ construct with the TM domain of MycP$_5$ resulted in full restoration of the ESX-5 complex to WT levels (Fig. 4*B*, *lane 7, P.P1-TM5*). In conclusion, our results show that the crucial and system-specific role of both the protease and TM domain of mycosins in ESX secretion is consistently linked to the stabilization of their respective ESX complex.

Discussion

Mycosin is an enigmatic component of the T7S system, as it is a highly conserved and essential component of this system, but its protease activity is dispensable for secretion. Although we were previously able to assign the essential role of mycosins to stabilizing the secretion complex in the mycobacterial cell envelope, the specific mycosin domains involved in this stabilization remain unknown. Here we set out to obtain insight into the role of individual mycosin domains in this process by producing various domain deletions and hybrid mycosin constructs.

It has been shown previously that the N-terminal extension, called the propeptide in subtilisins, is not processed but remains tightly wrapped around the protease domain of mycosins (20, 21). In line with this, our results also strongly suggest that the extension solely has a structural role, as it is able to stabilize protease domains of mycosins from different ESX sys-

Protease domain and TM domain mediate MycP system specificity

tems (Figs. 1*B*, *lane 7*, *P5NE1*, and 2*B*, *lane 7*, *P1NE5*). This is further supported by a study by Sun *et al.* (22), who showed that, although the structural conformation of the protease domain of MycP$_1$ is not altered by deleting the extension, molecular dynamics simulations did show increased flexibility, suggesting decreased stability of the protease domain. This can be explained by the presence of stabilizing interactions between these two domains, *i.e.* an anti-parallel β-sheet and a disulfide bond. The decreased stability is apparently more severe during *in vivo* expression in *M. marinum* (Figs. 1*B*, *lane 5*, *P5ΔNE*, and 2*B*, *lane 5*, *P1ΔNE*) compared with the MycP$_1$ purified from *Escherichia coli* by Sun *et al.* (22).

Our results clearly show that both the protease and TM domain are essential and function in a system-specific manner in ESX-mediated secretion and stabilization of the ESX complex (Figs. 1, 2, and 4). However, the importance and role of the TM domain appears to be complicated. Although it is clearly required for secretion and ESX-5 complex stability, the hybrid MycP$_5$ construct with the TM domain of MycP$_1$ (P5TM1) did remove the requirement for MspA in the *mycP$_5$* knockout, suggesting that the TM domain of MycP$_1$ is able to take over the role of the equivalent domain of MycP$_5$ to a minor extent. A possible role of the TM domain is to properly localize mycosins to their respective ESX complex by a specific interaction with this membrane complex. Thus, we postulate that a small subpopulation of P5TM1 is still capable of interacting with the ESX-5 complex, possibly only by the protease domain, whereas TM1 only serves as a membrane anchor. This results in a very limited level of functional ESX-5 complexes, showing that this strain is capable of (very limited) growth in the absence of MspA. In addition to this, the expression levels of mycosins seem to be highly influenced by the presence and nature of the TM domain. Although its deletion strongly increased protein levels, exchange of TM domains in the hybrid constructs also affected protein levels; *e.g.* constructs with the TM domain of MycP$_5$ generally resulted in higher protein levels irrespective of the identity of the protease domain. Especially the increased stability of mycosin in the absence of a TM domain is intriguing, as it suggests that WT mycosin is a relatively unstable protein. The question is then whether a high turnover is somehow related to its function.

In contrast to the TM domain, the protease domain of MycP$_5$ is absolutely required for ESX-5–mediated secretion and stabilization of the ESX-5 membrane complex, a role that cannot be replaced by the protease domain of MycP$_1$. The essential role of the protease is not related to its protease activity, as we and others showed previously that active-site mutants in MycP$_1$ and MycP$_5$ are fully able to mediate secretion and membrane complex stability of the respective systems (11, 15). We therefore tried to further dissect the specific features of MycP$_5$ that are essential for ESX-5 secretion by deleting its distinctive extended loops in the protease domain. However, none of the loops appear to be involved in the essential and system-specific function. Notably, deletion of loop 2 results in a strong reduction of MycP$_5$ levels (Fig. 3*B*, *lane 5*), and as this is also the most strongly conserved loop (Table S1), we expect it to play a structural role in, *e.g.*, establishing a stable protein conformation. This is also supported by the observation that we were unable to

produce stable MycP$_1$ lacking loop 2 in *E. coli* and the presence of a cysteine residue, which forms a disulfide bridge with the core protease structure (20, 21). However, as we were still able to detect low levels of MycP$_5$ lacking loop 2 in *M. marinum*, we cannot exclude that it is involved in the system-specific functioning of MycP$_5$. In addition to this, although deleting loop 1 also resulted in lower expression levels and an ESX-5 secretion defect, MycP$_5$ with loop 1 of MycP$_1$ is fully functional, showing that this loop is not involved in the system-specific functioning of the protease. Deleting loop 1 in MycP$_1$ did not affect protease activity *in vitro*, suggesting that this loop is also not directly involved in substrate recognition. These combined results suggest that loop 1 and loop 2 have a general structural role in mycosins. In contrast, loop 3 and loop 5 can be removed in MycP$_5$ without affecting protease integrity and ESX-5 functionality. Interestingly, MycP$_5$ and MycP$_{P1}$ appear to be processed in the larger loop 5, which is not conserved in all mycosins (*e.g.* Fig. S4). The two resulting MycP fragments are expected to form a single stable unit because of the structural requirement of the N-terminal extension (Figs. 1*B*, *lane 5*, *P5ΔNE*, and 2*B*, *lane 5*, *P1ΔNE*). The observed cleavage is not an autoproteolytic process; it is not dependent on either proteolytic active MycP$_5$ or on a functional ESX-5 system, as we also observed this processing when we expressed HA-tagged MycP$_5$ and a corresponding HA-tagged active site mutant in *M. smegmatis* that lacks ESX-5 (Fig. S3*C*). In addition, for the MycP$_5$ construct with the TM domain deletion, some full-length protein could be observed, showing that the involved protease is not able to fully process this truncate, either because of the high overexpression or because this construct does not localize to the inner membrane. Although we cannot exclude that loop 5 is required for full functionality of the putative protease activity of MycP$_5$, it is remarkable that this loop is only present in a subset of MycP proteins, including MycP$_2$ and mycosins from plasmid-borne ESX systems (25).

The observation that the hybrid construct with the protease domain of MycP$_{P1}$ (Fig. 3, *B*, *lane 9*, *P.P1-TM5*, and *C*, *lane 18*) of the ESX-P1 system and the TM domain of MycP$_5$ also results in successful complementation of the *mycP$_5$* knockout is not very surprising, considering the high similarity of the two protease domains (Fig. S4 and Table S1). Interestingly, WT MycP$_{P1}$ was not able to complement the *mycP$_5$* knockout, whereas its presence did result in a small increase in ESX-5 complex levels. ESX-5–mediated secretion was not observed, nor did this complementation allow the bacterium to grow in the absence of MspA. Although this again suggests an important role of the TM domain in the system-specific functioning of MycP, we cannot exclude that the observed different expression levels of the proteases are responsible for the different phenotypes.

Finally, it is interesting to note that almost all tested constructs resulted in an "all-or-nothing" phenotype regarding secretion. Except for the partial complementation of P5TM1, which resulted in a strongly reduced growth rate of the bacterium in the absence of MspA, secretion was either present or absent in the complemented knockout strains of *mycP$_1$* and *mycP$_5$*. This is especially surprising, as we observed a high variability in protein levels of the HA-tagged (hybrid or truncated) *mycP$_1$* and *mycP$_5$* constructs. Apparently, only small amounts

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of mycosin are required to allow efficient secretion via the ESX-1 and the ESX-5 membrane complex. Because we have good indications that mycosins have a role in the stability of the membrane complex (11), this could also imply that each bacterium needs only a small number of active secretion machineries to secrete substantial amounts of substrate. Furthermore, we also observed that none of the successful complementations of Δ*mycP₁* resulted in a difference in the processing of EspB, although this is not surprising, as we did not alter the catalytic site in any of the *mycP₁* constructs.

Our combined results show that, instead of a highly defined region, a major portion of mycosin, comprising both the protease and the TM domain, is involved in the essential role of this component in ESX secretion, *i.e.* in the stabilization of the respective ESX complex (for an overview, see Table S2). As the protease domain of $MycP_1$ is not functionally redundant to $MycP_5$, opposed to the protease domain of MyP_{P1}, this provides a starting point for future studies to identify the regions and residues in the mycosin protease domain that are involved in interacting with and stabilizing their respective ESX complex.

Experimental procedures

Bacterial strains and culture conditions

M. marinum M (26) was used for all *M. marinum* experiments. The *mycP₁* and *mycP₅* knockout mutants of this strain have been described previously by Ates *et al.* (6) and van Winden *et al.* (11). *M. marinum* was grown at 30 °C on 7H10 agar with 10% Middlebrook OADC (BD Biosciences) or in Middlebrook 7H9 liquid medium with 10% Middlebrook ADC and with 0.05% Tween 80 at 30 °C and 150 rpm. *M. smegmatis* MC²155 was used for all *M. smegmatis* experiments and was grown at 37 °C on 7H10 agar with 10% Middlebrook OADC or in Luria-Bertani (LB) liquid medium supplemented with 0.05% Tween 80 at 150 rpm. *E. coli* strains DH5α and Rosetta were grown at 37 °C on LB agar or in LB liquid medium at 37 °C and 200 rpm. Culture media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin, 25 μg ml^{-1}; hygromycin, 50 μg ml^{-1}; streptomycin, 35 μg ml^{-1}; ampicillin, 100 μg ml^{-1}.

Cloning

The *mycP₁* and *mycP₅* *M. marinum* genes were amplified from *M. marinum* M genomic DNA and *mycP_{P1}* from *M. marinum* E11 genomic DNA by PCR with anchored primers (EcoRI and HindIII; Table S3). The active site mutant of *mycP₅* has been created in a previous study (11). The hybrid combinations of *mycP₁*, *mycP₅*, and *mycP_{P1}* point mutations and the HA tag were introduced with nested primers (Table S3). All generated constructs were subsequently digested by EcoRI and HindIII and ligated into similarly digested pMV361 (27). The plasmids used to express $MycP_{1mth}$ and $EspB_{mtb}$ have been described previously by Wagner *et al.* (21). The *mycP1_{mth}* construct was modified with anchored (NdeI and XhoI) and nested primers (Table S3) to create *mycP_{mth}*Δloop1, which was ligated into pET-28a following NdeI and XhoI digestion. All generated plasmids were verified by sequencing of the cloned inserts.

M. marinum protein secretion and BN-PAGE analysis of ESX-5 complex

For protein secretion analysis, *M. marinum* strains were grown in 7H9 liquid medium supplemented with 10% Middlebrook ADC and 0.05% Tween 80 until mid-logarithmic phase (A_{600} of 1–1.4), and then the cells were washed and inoculated in 7H9 liquid medium containing 0.2% dextrose, 0.2% glycerol, and 0.05% Tween 80 at an A_{600} of 0.5. After 16 h of growth, the cells were pelleted (10 min at 3000 × g), washed with PBS, and resuspended in SDS loading buffer (pellet fraction). Supernatants were passed through an 0.45-μm filter, and the proteins were precipitated with TCA, washed with acetone, and resuspended in SDS loading buffer (supernatant fraction). Alternatively, cell surface proteins were extracted from pelleted cells with 0.5% Genapol X-080 as described previously (11), the cells were repelleted and resuspended in SDS loading buffer (Genapol pellet fraction), and concentrated SDS loading buffer was added to the Genapol X-080–containing supernatant (Genapol supernatant fraction). Proteins from the various fractions were separated on SDS-PAGE gels (with 10% to 16% acrylamide, depending on the size of the analyzed proteins), transferred to a nitrocellulose membrane, and stained with the appropriate antibodies (see below). For both the secretion analysis and the HA expression blots, at least three colonies of each strain were tested. The immunoblots used are an accurate representation of the obtained, consistent results.

For ESX-5 complex analysis, *M. marinum* was grown to an A_{600} of 1.2–1.5, pelleted, and resuspended in PBS with 250 mM sucrose. Cells were lysed with a One-Shot Cell disruptor (Constant Systems Ltd.), and unbroken cells were pelleted at 3000 × g. The cell envelope (CE) fraction was separated from the soluble fraction by centrifugation at 200,000 × g for 45 min, and the CE fraction was subsequently resuspended in PBS with 250 mM sucrose. Membrane proteins were extracted from the CE fraction with the mild detergent dodecyl maltoside at a concentration of 0.25%. Solubilized protein (complexes) was either separated under native conditions on a 3–12% NativePage Novex BisTris Protein Gel (Life Technologies) and transferred to a polyvinylidene difluoride membrane or under denaturing conditions on a 12.5% SDS-PAGE gel, which was subsequently transferred to a nitrocellulose membrane. Blots were stained with anti-GroEL2 (Cs44; John Belisle, National Institutes of Health, Bethesda, MD), anti-PE_PGRS (26), anti-EsxA/ESAT-6 (Mab Hyb76-8), anti-HA (HA.11, Covance, Biolegend), anti-EccB₅ (12, Colorado State University), anti-EspB (EPFL, Lausanne, Switzerland), anti-EccB₁ (11), or anti-FtsH (28) antibodies. Apart from the monoclonal anti-PE_PGRS and anti-EsxA preparations, these are polyclonal rabbit antisera.

Protein expression, purification, and activity assays

His-tagged $EspB_{mtb}$ and $MycP_{1mth}$ were expressed and purified from *E. coli* Rosetta (DE3) as described previously by van Winden *et al.* (11). $MycP_1$ activity assays were performed in 20 mM HEPES (pH 7.5), 100 mM at 37 °C for 20 h with 0.15 mg ml^{-1} $MycP_1$, and 0.25 mg ml^{-1} EspB. Reactions were stopped by addition of SDS sample buffer and incubation at 94 °C for 5

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min. Proteins were separated on a 12.5% SDS-PAGE gel and visualized by Coomassie staining.

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Protease domain and transmembrane domain of the type VII secretion mycosin protease determine system-specific functioning in mycobacteria

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