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Vincent J. C. van Winden  
*VU University Medical Center, The Netherlands*

Roy Ummels  
*VU University Medical Center, The Netherlands*

Sander R. Piersma  
*VU University Medical Center, The Netherlands*

Connie R. Jiménez  
*VU University Medical Center, The Netherlands*

Konstantin V. Korotkov  
*University of Kentucky, kkorotkov@uky.edu*

See next page for additional authors

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Mycosins Are Required for the Stabilization of the ESX-1 and ESX-5 Type VII Secretion Membrane Complexes

Vincent J. C. van Winden, a Roy Ummels, a Sander R. Piersma, b Connie R. Jiménez, b Konstantin V. Korotkov, c Wilbert Bitter, a,d Edith N. G. Houben d

Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands; Department of Medical Oncology, OncoProTeomics Laboratory, VU University Medical Center, Amsterdam, The Netherlands; Department of Molecular and Cellular Biochemistry, and Center for Structural Biology, University of Kentucky, Lexington, Kentucky, USA; Section Molecular Microbiology, Amsterdam Institute of Molecules, Medicines and Systems, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

ABSTRACT Pathogenic mycobacteria contain up to five type VII secretion (T7S) systems, ESX-1 to ESX-5. One of the conserved T7S components is the serine protease mycosin (MycP). Strikingly, whereas MycP is essential for secretion, the protease activity of MycP1 in Mycobacterium tuberculosis has been shown to be dispensable for secretion. The essential role of MycP therefore remains unclear. Here we show that MycP1 and MycP5 of M. marinum have similar phenotypes, confirming that MycP has a second unknown function that is essential for its T7S system. To investigate whether this role is related to proper functioning of the T7S membrane complex, we first analyzed the composition of the ESX-1 membrane complex and showed that this complex consists of EccBCDE, similarly to what was previously shown for ESX-5. Surprisingly, while mycosins are not an integral part of these purified core complexes, we noticed that the stability of both the ESX-1 complex and the ESX-5 complex is compromised in the absence of their MycP subunit. Additional interaction studies showed that, although mycosins are not part of the central ESX membrane complex, they loosely associate with this complex. We hypothesize that this MycP association with the core membrane complex is crucial for the integrity and functioning of the T7S machinery.

IMPORTANT Among the major virulence factors of pathogenic mycobacteria are the type VII secretion (T7S) systems. Three of these systems, ESX-1, ESX-3, and ESX-5, have been shown to be crucial for virulence or viability. Here we describe the function of mycosin proteases, which are conserved components within these systems. We show that MycP1 and MycP5 have a second, proteolytic-independent function which is essential for the T7S system. We additionally found that this second essential role is related to the stabilization and proper functioning of their respective ESX membrane core complexes. Finally, we found that this is mediated by a loose association of MycP with the complex. Understanding the essential role of mycosins in type VII secretion systems, which play central roles in the virulence and viability of pathogenic mycobacteria, may provide new intervention strategies to treat tuberculosis.

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Address correspondence to Edith N. G. Houben, e.n.g.houben@vum.nl.

Pathogenic mycobacteria such as Mycobacterium tuberculosis and Mycobacterium leprae remain notorious human pathogens. Important virulence factors of pathogenic mycobacteria are the type VII secretion (T7S) systems and their substrates, which are required for the completion of the macrophage infection cycle and the uptake of nutrients and metabolites across its exceptionally hydrophobic and impermeable cell envelope (CE) (1–4). Pathogenic mycobacteria have up to five of these systems, called ESX-1 to ESX-5, of which ESX-1, ESX-3, and ESX-5 have been shown to be essential for virulence or bacterial viability (1, 5, 6).

ESX-1 is of pivotal importance for the virulence of pathogenic mycobacteria, with ESX-1 substrates being linked to phagosomal escape by destabilizing the phagosomal membrane of macrophages (1, 7). The importance of the ESX-1 system for virulence is also shown by the absence of part of the esx-1 genomic locus in the vaccine strain Mycobacterium bovis BCG (8–10). This deletion is the major determinant for the attenuation of this strain. Also, in the fish pathogen Mycobacterium marinum, a close relative of M. tuberculosis, ESX-1 has been shown to mediate phagosomal escape and deletion of the esx-1 region leads to a strong attenuation in zebrafish (11, 12).

The most recently evolved mycobacterial T7S system, ESX-5, is present only in the cluster of slow-growing mycobacteria. Interestingly, this cluster contains most of the pathogenic species. ESX-5 is responsible for the secretion of many proteins of the so-called proline-glutamic acid (PGE) and proline-proline-glutamic acid (PPGE) families and is linked to host immune modulation. In addition, ESX-5 has been shown to be essential for in vitro growth of M. marinum and M. bovis BCG by permeating the outer membrane to allow nutrient uptake (4, 13–15).

The ESX systems of mycobacteria share a set of conserved components (16, 17), five of which have one or more predicted transmembrane domains and are cell envelope localized (2). Four of these membrane proteins of the ESX-5 system, i.e., EccB5 to EccE5,
form a large membrane complex of 1.5 MDa (2, 17, 18). Although crystal structures of the soluble domains of the individual components EccB, EccC, and EccD have been published previously (19, 20), there are currently no structural data for this complete membrane complex. Furthermore, the biochemical composition of this complex has been elucidated only for the ESX-5 system, whereas the composition and size of the other ESX complexes remain unknown.

The fifth conserved component with a predicted transmembrane domain is the subtilisin-like protease mycosin (MycP), which is among the most conserved T7S components (21). Although previous pulldown experiments indicated that MycP is not part of the core ESX membrane complex, MycP1 and MycP5 have been shown to be essential for mycobacterial viability and MycP1, and MycP5 are essential for ESX-1- and ESX-5-associated secretion, respectively (4, 22, 23). This indicates that each MycP is essential for and functions specifically within its respective ESX system. The crystal structures of the protease domains of MycP1 and MycP3 show a highly conserved overall subtilisin-like structure, with differences in the substrate binding groove indicating different substrate specificities (24, 25).

Surprisingly, thus far, only one substrate, ESX-1 substrate EspB, is known for any of the mycosins. This protein is processed by MycP1, in vitro and upon secretion by M. tuberculosis (22). Importantly, proteolytic activity of MycP1 is, however, not essential for ESX-1-associated secretion; a catalytically inactive MycP1 mutant of M. tuberculosis even showed increased secretion of ESX-1 substrates (22). Therefore, the essential function of mycosins in secretion remains unknown. The catalytically inactive MycP1 mutant of M. tuberculosis additionally showed decreased virulence in mice, but it is still unknown whether this is a direct effect of the deficiency in EspB processing or due to the observed increased secretion of ESX-1 substrates in this mutant. Together, these observations suggest a dual role of MycP within T7S, with MycP1 being essential for ESX-1 secretion whereas proteolytic activity of MycP1 is not essential for this function.

To further elucidate the dual function of mycosin proteases, we investigated MycP1 and MycP3 functioning in M. marinum. We show that, similarly to the ESX-1 system in M. tuberculosis, ESX-1- and ESX-5-mediated secretion is independent of the (predicted) proteolytic activity of their respective mycosins. However, we show that both the ESX-1 and ESX-5 membrane complexes are not stable in the absence of MycP1 and MycP3, respectively, providing an explanation of why mycosins are essential components in the T7S system.

RESULTS

MycP1 is essential for ESX-1-dependent secretion in M. marinum, while its protease activity is not. To confirm the dual role of MycP1 in another species (22), we first deleted the mycP1 gene of M. marinum via allelic exchange and confirmed that the gene was successfully deleted via PCR analysis (unpublished observation). As expected, this knockout mutant was no longer able to secrete the ESX-1 substrates EsxA, EsxB, and EspB. All examined substrates were still detected in the pellet fractions (Fig. 1A). The ESX-1 substrate EspE was also no longer extractable from intact cells by the mild detergent Genapol X-080 (Fig. 1B), indicating that it was no longer present on the cell surface. ESX-1-dependent secretion was fully complemented by the introduction of the wild-type gene (P1; Fig. 1A and B). To assess the role of the protease activity of MycP1 in secretion, we complemented the ΔmycP1 mutant also with a proteolytically inactive version, mycP1::S354A (PISA). In agreement with a previous study (22), expression of proteolytic inactive MycP1 resulted in increased secretion of EsxA and EspB (Fig. 1A). We also observed an increase in the amount of surface-localized EspE (Fig. 1B). Whereas the wild-type strain showed efficient processing of EspB, mainly full-length EspB was detected in the supernatant of the S354A mutant (Fig. 1A), which is consistent with the effect observed for the MycP1 active site mutant of M. tuberculosis (22). There was no increase in the ESX-5-dependent secretion of proteins of the PE subfamily with polymorphic GC-rich repetitive sequences (PE_PGRS), showing that the proteins are not in general more efficiently secreted by the MycP1 proteolytically inactive mutant.

The secretion of ESX-1 substrates has been shown to be essential for contact-dependent lysis of erythrocytes by M. marinum, which serves as a model for the ESX-1-dependent lysis of phagosomal membranes and thus for mycobacterial virulence (11). We confirmed that our wild-type M. marinum strain was capable of lysing erythrocytes, whereas the ΔmycP1 mutant showed no hemolytic activity (Fig. 1D). We could restore this lysing capability by complementing the mutant with both wild-type mycP1 and the proteolytically inactive MycP1 mutant. The latter complementation resulted in significantly increased hemolysis activity compared to that seen with wild-type cells, which is in line with the increased secretion of ESX-1 substrates in this mutant. Together, these data show that, in the presence of a proteolytically inactive MycP1 variant, the ESX-1 system is more active. Finally, we created a version of MycP1, where the access to the active site is partially blocked by placing a bulky amino acid, i.e., a tyrosine, at different positions in the substrate binding groove. We first analyzed the effect of these mutations on the ability of MycP1 of Mycobacterium thermoresistibile (MycP1th) to cleave its substrate EspB in vitro (25).Introducing a tyrosine at position 239, generating mycP1th::N239Y, completely blocked protease activity (Fig. 1C). Next, we investigated the effect of the mycP1th::N239Y mutation (N239Y in M. marinum) on secretion and hemolysis by M. marinum. The bulky mutant (P1Bulky) showed a phenotype similar to that of the active site mutant (Fig. 1A, B, and D), with the bacteria still capable of oversecreting ESX-1 substrates and a more efficient lysis of erythrocytes. This indicates that in addition to protease activity, substrate binding to MycP1 is also not essential for secretion.

MycP3 shows a phenotype similar to that of MycP1. Ates et al. (4) previously showed that a mycP3 transposon mutant in M. marinum is no longer able to secrete the ESX-5 substrate group of PE_PGRS proteins. In this study, we confirmed this secretion defect for an M. marinum mycP3 knockout strain (Fig. 2) and that the original phenotype could be restored by complementation with wild-type mycP3 (P5; Fig. 2). Similarly to the phenotype of MycP1, complementation of the mycP3 knockout strain with the predicted proteolytically inactive mutant mycP3::S461A (P5SA) or the bulky mutant mycP3::D362Y (P5Bulky) fully restored ESX-5-dependent secretion. However, we did not observe an increase in the secretion of ESX-5 substrates, such as the PE_PGRS proteins or EsxN and LipY. Importantly, LipY, which is normally processed upon secretion (26), was processed in the active site and bulky mutants in a manner similar as seen in the wild-type strain. Also, the pattern seen with the PE_PGRS proteins, which are potentially processed upon secretion, was unaltered. Either MycP3 is not in
involved in the processing of these substrates or there is redundancy in the protease activities. In conclusion, MycP5 is also essential for protein secretion via ESX-5, but this function is not linked to its putative protease activity.

The composition of the ESX-1 membrane complex is similar to that of the ESX-5 membrane complex. Because MycP is probably an inner membrane protein, we hypothesized that MycP may be involved in the correct functioning of the core membrane complex of T7S systems. We have shown previously by blue native PAGE (BN-PAGE) and antibody (Ab) pulldown experiments that the membrane complex of the ESX-5 system has a size of 1.5 MDa and consists of four conserved membrane proteins, i.e., EccB5, EccC5, EccD5, and EccE5 (2); no MycP5 could be detected in these purified samples. Here, we set out to improve the purification procedure by the introduction of an affinity tag, not only to more accurately detect less-abundant components of the ESX-5 membrane complex but also to determine the composition of the ESX-1 membrane complex.

As the ESX-1 complex had not been analyzed before, we first investigated whether the ESX-1 system of M. marinum forms a similar complex. To analyze this, we generated polyclonal antibodies directed against the C-terminal fragment of EccB1. These antibodies were used to identify the ESX-1 membrane complex in n-dodecyl β-d-maltoside (DDM)-solubilized membrane proteins separated on BN-PAGE, as was done previously for ESX-5. EccB1 antibodies stained a number of different complexes, the largest of which was approximately 1.5 MDa, similarly to ESX-5 (Fig. 3A). To be able to isolate the complex, we introduced EccCb1 containing a C-terminal Twin-Strep-tag in an eccCb1 transposon mutant of M. marinum (27). The affinity tag did not interfere with EccB1 functioning, as introduction of the construct fully restored ESX-1-dependent secretion (see Fig. S1 in the supplemental material). The tag also did not interfere with formation of the 1.5-MDa ESX-1 membrane complex as shown by BN-PAGE analysis of detergent-solubilized membrane fractions and immunoblotting using Strep antibodies (Fig. 3A), although these complexes were less pronounced (Fig. 3A).

We subsequently performed pulldown experiments on detergent-solubilized membrane fractions using Strep-Tactin beads. We observed a number of copurified proteins after Coomassie staining (Fig. 3B), and we confirmed that two were EccB1,-Strep and EccB3 by immunoblot analysis (Fig. 3C). To identify the other copurified proteins, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on the complete
Strep-tag-purified sample. This analysis showed the significantly increased presence of EccB1, EccCa1, EccCb1, EccD1, EccE1, and a hypothetical protein, MMAR_2712 (Table 1), in the samples containing EccCb1-Strep but not in control samples containing purified material from solubilized membranes of wild-type M. marinum. The presence of MMAR_2712 was surprising and might indicate the presence of an additional component. However, homology and structure predictions (Phyre2) indicated that MMAR_2712 is a transmembrane protein with a large periplasmic domain containing a predicted phosphate binding site, an activity unrelated to ESX-1 functioning. Furthermore, the gene encoding this protein is highly conserved in many bacterial species without an ESX-1 system. To test a possible interaction of this protein with the ESX complex, we introduced an N- or a C-terminal hemagglutinin (HA) tag in MMAR_2712 and isolated this protein using HA antibody beads. Subsequently, we used immunoblotting to determine if ESX-1 components were copurified. However, this experiment failed to confirm any interaction of MMAR_2712 with ESX-1 components (unpublished observations). From these combined observations, we conclude that it is unlikely that MMAR_2712 is a component of ESX-1. As with the previously analyzed ESX-5 membrane complex, we were not able to detect significantly more MycP5 peptides in the purified ESX-1 substrates with anti-PE_PGRS and anti-EsxN. As a loading control, blots were probed with antibodies directed against the ESX-1 substrate EsxA and cytosolic GroEL2.

MycP1 and MycP5 are involved in the stability of the ESX membrane complexes. Although mycosins do not appear to be part of the core complex, the mycosins might still be involved in the correct functioning of this membrane complex. To analyze this, we first analyzed the presence of the ESX-5 membrane complex in the absence or presence of MycP5. While the 1.5-MDa membrane complex was readily visualized on BN-PAGE using polyclonal antibodies directed against EccB5, EccC5, and EccD5, as shown by immunoblot analysis and LC-MS/MS analysis (Table 2). The mass spectrometry analysis revealed that, in this preparation also, there were no additional proteins copurified with EccC5; although spectral counts for MycP5 could be detected, these numbers were not above the spectral count threshold levels (Table 2). We therefore conclude that the mycosins are probably not a stable integral part of the ESX membrane complex.

Next, we also modified the ESX-5 system with a Twin-Strep-tag to allow more-efficient purification of the ESX-5 membrane complex. For this, we complemented the previously characterized M. marinum eccC5 knockout strain (4) with EccC5 containing a C-terminal Twin-Strep-tag. This affinity tag did not interfere with ESX-5-dependent secretion (see Fig. S1 in the supplemental material) or with formation of the 1.5-MDa ESX-5 membrane complex (Fig. 3). The Strep-tag purification of EccC5-Strep was significantly more efficient than the EccCb1-Strep purification and resulted in the copurification of the three known interactors, i.e., EccB5, EccD5, and EccE5, as shown by immunoblot analysis and LC-MS/MS analysis (Table 2). Although spectral counts for MycP5 could be detected, these numbers were not above the spectral count threshold levels (Table 2). We therefore conclude that the mycosins are probably not a stable integral part of the ESX membrane complex.

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Complementation with both wild-type mycP$_5$ and mycP$_5$::S461A restored the complex to wild-type levels. This effect on complex formation was not due to decreased stability of the separate subunits, as the expression levels of EccC$_5$ and EccD$_5$ were not affected, whereas EccB$_5$ levels were reduced only slightly upon the mycP$_5$ deletion. A similar effect was observed for the ESX-1 complex; we could detect the 1.5-MDa ESX-1 complex in the wild-type strain, while this complex was not observed in the absence of MycP$_1$ (Fig. 4B). This phenotype could be complemented by introduction of wild-type MycP, or the active site mutant. Expression levels of EccB$_1$ were not affected by knocking out mycP$_5$. These results suggest that the strongly reduced membrane complex levels in the absence of the respective mycosins were not due to diminished expression of individual membrane components.

The observation that small amounts of the 1.5-MDa ESX-5 membrane complex could be detected in the mycP$_3$ knockout strain suggested that the membrane complex is less efficiently formed or is less stable. To distinguish between these two possibilities, we treated half of the membrane fractions with the cross-linking agent dithiobis(succinimidyl propionate) (DSP) before solubilization was performed to fix protein-protein interactions. DSP cross-linking did not affect ESX-5 complex levels in the wild-type strain. Also, in the negative-control strain, i.e., the eccC$_5$ knockout mutant, the presence of DSP did not restore complex formation of the remaining components. In contrast, cross-linking had a major effect in the mycP$_3$ mutant, as the ESX-5 membrane complex was detectable at wild-type levels in the DSP-treated membranes (Fig. 4C). A similar experiment was performed for ESX-1. Also, in this case DSP treatment of mycP$_5$ knockout membranes resulted in a stabilizing effect on the ESX-1 complex (see Fig. S2B in the supplemental material). This shows that the conserved components of the ESX-1 and ESX-5 membrane complex interact in principle and seem to properly form the ~1.5-MDa membrane complex in the absence of their MycP component but that the complexes more easily dissociate after detergent extraction. We therefore conclude that the mycosins of ESX-1 and ESX-5 are involved in stabilization of the respective membrane complexes. We propose that this stabilization is crucial for membrane complex functioning, explaining the essential role of MycP in the T7S system.

**MycP$_5$ is associated with the ESX-5 core complex.** In the mass spectrometry results from the purified EccCb$_1$ and EccC$_5$ protein samples, we did observe a few specific spectral counts for MycP$_1$ and MycP$_5$, respectively. These counts were too low to conclude that MycP is a stable component of the T7S membrane complex. However, we hypothesized that MycP could be loosely associated

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**TABLE 1 Proteins copurified with EccCb$_1$-strept**

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>Protein description</th>
<th>MW</th>
<th>Sequence coverage (%)</th>
<th>MS/MS normalized spectral count</th>
<th>Fold change</th>
<th>P value</th>
<th>NSAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>EccCa$_1$</td>
<td>ESX-1 core component</td>
<td>80.8</td>
<td>73.5</td>
<td>WT: A 20, B 30</td>
<td>eccCb$_1$-strept: A 271, B 157</td>
<td>19.0</td>
<td>2.5 x 10$^{-3}$</td>
</tr>
<tr>
<td>EccCb$_1$</td>
<td>ESX-1 core component</td>
<td>64.6</td>
<td>49.2</td>
<td>WT: A 7, B 172</td>
<td>eccCb$_1$-strept: A 98, B 98</td>
<td>37.5</td>
<td>2.4 x 10$^{-3}$</td>
</tr>
<tr>
<td>EccB$_1$</td>
<td>ESX-1 core component</td>
<td>51.3</td>
<td>75.5</td>
<td>WT: A 7, B 85</td>
<td>eccCb$_1$-strept: A 53, B 53</td>
<td>13.9</td>
<td>1.4 x 10$^{-3}$</td>
</tr>
<tr>
<td>EccE$_1$</td>
<td>ESX-1 core component</td>
<td>50.9</td>
<td>64.7</td>
<td>WT: A 11, B 76</td>
<td>eccCb$_1$-strept: A 46, B 46</td>
<td>10.1</td>
<td>4.6 x 10$^{-3}$</td>
</tr>
<tr>
<td>MMAR_2712</td>
<td>Hypothetical protein</td>
<td>76.1</td>
<td>54.1</td>
<td>WT: A 9, B 70</td>
<td>eccCb$_1$-strept: A 44, B 44</td>
<td>11.0</td>
<td>3.0 x 10$^{-3}$</td>
</tr>
<tr>
<td>EccD$_3$</td>
<td>ESX-1 core component</td>
<td>51.3</td>
<td>14</td>
<td>WT: A 0, B 18</td>
<td>eccCb$_1$-strept: A 11, B 11</td>
<td>$\infty$</td>
<td>9.7 x 10$^{-4}$</td>
</tr>
<tr>
<td>MycP$_1$</td>
<td>ESX-1 core component</td>
<td>47.7</td>
<td>41.7</td>
<td>WT: A 0, B 11</td>
<td>eccCb$_1$-strept: A 8, B 8</td>
<td>$\infty$</td>
<td>1.2 x 10$^{-3}$</td>
</tr>
</tbody>
</table>

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$^a$ LC-MS/MS was performed on Strept-tag-purified material from *M. marinum* wild-type (negative control) and *M. marinum*-eccCb$_1$-tn-eccCb$_1$-strept cell envelope fractions, followed by a two-way analysis. Proteins that showed $>$10 normalized spectral counts in both eccCb$_1$-strept pulldown samples and a normalized spectral abundance factor (NSAF) of $>$0.02 were selected. Data in columns A and B represent results from biological replicates. MW, molecular weight.
with the complex and could thereby stabilize the core complex. To investigate this, we tried to preserve this interaction by testing different mild detergents to solubilize the cell envelope proteins of the mycP5 deletion strain complemented with MycP5 containing a C-terminal HA tag for detection. The HA tag did not interfere with ESX-5-dependent secretion and therefore did not affect MyP5 functioning (see Fig. S1C in the supplemental material). Although several detergents did show solubilization compara-

### Table 2: Proteins copurified with EccC5-strep

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>Protein description</th>
<th>MW</th>
<th>Sequence coverage (%)</th>
<th>MS/MS normalized spectral count</th>
<th>Fold change</th>
<th>P value</th>
<th>NSAF</th>
</tr>
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<tbody>
<tr>
<td>EccC5</td>
<td>ESX-5 core component</td>
<td>152.6</td>
<td>71.4</td>
<td>WT A: 75, B: 50, eccC5-strep A: 706, B: 651</td>
<td>10.8</td>
<td>5.7 × 10⁻⁶</td>
<td>0.42</td>
</tr>
<tr>
<td>EccD5</td>
<td>ESX-5 core component</td>
<td>53.5</td>
<td>29.2</td>
<td>WT A: 24, B: 15, eccC5-strep A: 118, B: 111</td>
<td>5.9</td>
<td>4.4 × 10⁻³</td>
<td>0.20</td>
</tr>
<tr>
<td>EccE5</td>
<td>ESX-5 core component</td>
<td>44.0</td>
<td>57.6</td>
<td>WT A: 17, B: 8, eccC5-strep A: 103, B: 110</td>
<td>8.5</td>
<td>4.3 × 10⁻³</td>
<td>0.21</td>
</tr>
<tr>
<td>EccB5</td>
<td>ESX-5 core component</td>
<td>54.1</td>
<td>55.4</td>
<td>WT A: 22, B: 16, eccC5-strep A: 95, B: 84</td>
<td>4.6</td>
<td>8.3 × 10⁻³</td>
<td>0.16</td>
</tr>
<tr>
<td>MycP5</td>
<td>ESX-5 component</td>
<td>59.8</td>
<td>29.5</td>
<td>WT A: 0, B: 3, eccC5-strep A: 9, B: 19</td>
<td>6.9</td>
<td>4.8 × 10⁻³</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*LC-MS/MS was performed on Strep-tag-purified material from M. marinum wild-type (negative control) and M. marinum–eccC5–eccC5-strep cell envelope fractions, followed by a two way analysis. Proteins that showed >10 normalized spectral counts in both eccC5-strep pulldown samples and a normalized spectral abundance factor (NSAF) of >0.05 were selected. The NSAF was calculated by dividing the normalized spectral counts from the nanoLC-MS/MS experiment by the relative molecular weight (M_r) to obtain the spectral abundance factor (SAF) for each protein. Subsequently, each SAF was normalized by dividing it by the sum of the SAFs of the proteins in the complex. Data in columns A and B represent results from biological replicates. MW, molecular weight.
ble to that seen with DDM, they did not preserve the interaction of the ESX-5 complex with MycP$_5$ (Fig. 4E). Next, we tried to fix the interaction by treating the cell envelope fractions with DSP prior to the detergent extraction. Interestingly, the DSP treatment resulted in a shift of the HA-tagged MycP$_5$ to a molecular weight corresponding to the ESX-5 core complex, indicating that MycP$_5$ is associated with the ESX-5 complex after cross-linking. To confirm that cross-linking stabilizes the interaction, we performed Strept-Tactin pulldown experiments on cross-linked and detergent-solubilized membrane fractions of either the wild-type strain or a mycP$_5$ deletion strain complemented with MycP$_5$ containing a Twin-Strep-tag at its C terminus. Also, this tag did not interfere with MycP$_5$ functioning (see Fig. S1D). As we observed that DSP severely interfered with the pulldown, we used formaldehyde to fix protein-protein interactions. This cross-linking agent did not affect the pulldown efficiency, as similar MycP$_5$ levels were detected in the elution samples containing the Streptagged MycP$_5$, whereas no MycP$_5$ was detected in the elution of the wild-type samples by immunoblotting and Coomassie staining (see Fig. 4D; see also Fig. S3). The purification of MycP$_5$ resulted in the copurification of EccB$_5$ and EccE$_5$, albeit at relatively low levels (Fig. 4D). This interaction could be stabilized by formyl-aldehyde treatment, as we detected higher levels of EccB$_5$ and EccE$_5$ in the cross-linked samples (Fig. 4D). We did not detect the unrelated FtsH membrane component or the abundant GroEL2 cytosolic component in the elution samples (Fig. 4D). We did observe a very-low-intensity signal for EccB$_1$ in the elution fraction; however, in contrast to EccB$_5$ and EccE$_5$, this signal was reduced in the cross-linked elution sample, indicating that this represented nonspecific contamination of the eluate. Our data therefore seem to confirm that MycP$_5$ indeed interacts with the ESX-5 core complex components and that this interaction is essential for the stability and functionality of the ESX complexes corresponding to their respective mycosins.

DISCUSSION

In this study, we showed that the active site mutant of MycP$_1$ has a phenotype in *M. marinum* that is similar to that previously observed in *M. tuberculosis* (22). In that previous study, Ohol et al. (22) described a regulatory role of the proteolytic activity of MycP$_1$ in *M. tuberculosis*, with increased secretion by the MycP$_1$ active site mutation. This mechanism appears to be a conserved feature, as we also observed increased secretion of EsxA, EsxB, and EspE in an *M. marinum* strain harboring a proteolytically inactive MycP$_1$.

We used the ability of *M. marinum* to lyse erythrocytes in an ESX-1-dependent manner, to further analyze ESX-1 functioning. While the *M. marinum* ΔmycP$_1$ mutant was indeed unable to lyse erythrocytes, the active site mutant showed significantly increased hemolytic activity, corresponding to the increased activity of the ESX-1 system. It is possible that this was due to the increased secretion of EsxA, as this substrate has been indicated to be responsible for the hemolytic activity (11, 12, 28), although the other substrates of ESX-1 are also secreted in larger amounts. The disparity between the increased membrane lysing capability observed in *M. marinum* and the decreased virulence of *M. tuberculosis* in mice (22) may be explained by the immunogenicity of EsxA, which might result in reduced virulence in later stages of infection (29, 30).

MycP$_5$ showed a phenotype similar to that of MycP$_1$, with the mutation in the predicted active site not affecting the secretion of ESX-5 substrates. However, we did not observe increased secretion in the mycP$_5$ active site mutant, supporting the suggestion that the observed phenotype of the proteolytically inactive MycP$_1$ is caused by a specific MycP$_5$ substrate, which could be EspB (22). We also did not observe any differences in the (possible) processing of ESX-5 substrates in the mycP$_5$ active site mutant compared to the wild-type *M. marinum* strain. This also means that there are currently no MycP$_5$ substrates known. Therefore, the possibility remains that MycP$_5$ is proteolytically inactive, although it contains all the features known to be essential for protease activity. We prefer the hypothesis that the phenotype of the active site mutant of mycP$_5$ is a result of functional redundancy between MycP$_5$ and other proteases, possibly other mycosins.

We also investigated whether substrate binding is involved in the essential role of mycosins by introducing a bulky amino acid in the substrate binding pocket of MycP$_1$ and MycP$_5$. Because these modifications had an effect similar to that seen with the active site mutations, we can conclude that not only the proteolytic activity of mycosins but also substrate binding is not required for ESX-dependent secretion. It should be mentioned, though, that the mutated residue of MycP$_1$, N239, coordinates the oxyanion hole and, as such, may also affect proteolytic activity. Further experiments are required to determine whether EspB indeed cannot bind to MycP$_{inhA}^m$-N239Y.

To study the involvement of mycosin in T7S membrane complex functioning, we isolated both the ESX-1 membrane complex and the ESX-5 membrane complex using a Twin-Strep-tag that was fused at the C terminus of EccC. The Strept-tag purification considerably increased the yield and purity of the purified ESX-5 complex compared to the previous purifications using antibodies (2). Despite the improved purification, we were still unable to detect any additional (less-abundant) components; although a few spectral counts of MycP$_5$ were specifically detected with the EccC$_5$-Strep pulldown, these were not above the spectral count threshold levels. We also were unable to detect specific MycP$_5$ copurification by immunoblot analysis using MycP$_5$ antibodies. Also, in the Strept-pulldown experiments of the ESX-1 complex we could detect a few specific spectral counts for MycP$_5$, but these numbers were again below the threshold level.

We calculated the normalized spectral abundance factor (NSAF) of the Strept-tag-purified complexes using a method similar to a method described before (2) to estimate and compare the relative abundances of individual components of the ESX-1 and ESX-5 complexes. For this, the number of spectral counts (SpC) per isolated protein was divided by the protein’s length (L), which was again divided by the result of SpC/L for all isolated proteins in the experiment. This analysis revealed an EccC$_5$/EccB$_5$/EccE$_5$/EccD$_5$ ratio of approximately 2.1:1:1:1. This ratio is slightly different from the 2.2:1:2 ratio that was found for the antibody pulldown (2). It should be noted that EccC$_5$ might be overrepresented in the Strep pulldown results, as this component contains the affinity tag. The NSAF values of the ESX-1 purified proteins revealed a ratio of 9.7:4:4:1 for EccCa$_1$/EccCb$_1$/EccB$_1$/EccE$_1$/EccD$_1$, showing a similar distribution, in which the EccC subunits, which are produced as two separate proteins in ESX-1, are present at roughly double the amount seen with the other components. For ESX-1, only EccD$_1$ seemed to be underrepresented compared to ESX-5. This could suggest that the ESX-1 complex is less stable than the
ESX-5 complex, which could also explain the smearing pattern observed in BN-PAGE.

As MycP₁ or MycP₅ does not appear to be a (stable) component of the ESX-1 or ESX-5 complex in M. marinum, it was surprising that the presence of both MycP₁ and MycP₅ is required for complex stability. The instability of the ESX-5 complex in the mycP₁ knockout was further indicated by the observation that we could not stabilize the ESX-5 complex by cross-linking in the mycP₁ knockout background after repeated freeze-thaw cycles, while this was possible with wild-type samples (unpublished observations). The mechanical stress of this process is apparently surprising that the presence of both MycP₁ and MycP₅ is required for complex stability. The instability of the ESX-5 complex in the mycP₁ knockout was further indicated by the observation that we could not stabilize the ESX-5 complex by cross-linking in the mycP₁ knockout background after repeated freeze-thaw cycles, while this was possible with wild-type samples (unpublished observations). The mechanical stress of this process is apparently sufficient to dissociate this unstable complex. Using a cross-linking approach, we showed that the ESX-1 complex and the ESX-5 complex could be formed in the mycP₁ and mycP₅ knockout strains, respectively. Therefore, this indicates that the mycosins are associated with the complexes and are essential for their stability. Furthermore, this stabilization is required for the complex to be functional. The fact that we are unable to detect MycP₁ or MycP₅ above the threshold levels in the EccB₁ and EccC₅ pulldown experiments indicates that MycP associates with the membrane complex only loosely and that its interaction is not maintained after detergent extraction. This notion is supported by the observed shift of HA-tagged MycP₁ to a molecular weight corresponding to the ESX-5 complex on BN-PAGE, the detection of EccB₅ and EccE₅ in the elution samples from Strep-Tactin pull-down experiments using Strep-tagged MycP₅, and the fact that we observe increased amounts of copurified EccB₅ and EccE₅ after cross-linking. From this, we conclude that there is indeed an interaction between MycP₁ and the ESX-5 core complex, which could explain the observed effects on complex stability. Although we cannot explain the exact mechanism by which a loose association between MycP₁ and the core ESX complex can affect the stability of the complex, there are comparable effects known, as reported in the literature. In type IV pilus biogenesis in Neisseria meningitides, for instance, the outer membrane protein PilW stabilizes multimeric PilQ, the outer membrane secretin, even though PilW is not part of the multimeric complex formed by PilQ (31).

In summary, this study for the first time provided insight into the essential function of mycosins in the T7S system. We propose a new model for the T7S systems in mycobacteria, with the mycosins being associated with their respective membrane complexes, which is crucial for the full integrity of the core secretion complex (Fig. 5A). In the absence of mycosin, the complex is less stable and, as a result, nonfunctional (Fig. 5B).

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** M. marinum MUSA (32) was used for all M. marinum experiments unless stated otherwise. M. marinum wild-type strains and the various derived knockout mutants were grown on 7H10 agar supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) (BD Biosciences) at 30°C or in Middlebrook 7H9 liquid medium supplemented with 10% Middlebrook ADC and, when required, 0.05% Tween 80 at 30°C and 150 rpm. Escherichia coli strains were grown in Luria-Bertani (LB) liquid medium or on LB agar. Medium was 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; chloramphenicol, 30 μg ml⁻¹. E. coli strain DH5α was used for DNA cloning and plasmid accumulation and E. coli strain Rosetta for recombinant protein expression.

**Generating the mycP₁ knockout in M. marinum.** The generation of the mycP₁ and eccC₅ knockout strains that were used in this study was described previously by Ates et al. (4). Notably, a pSMT3 plasmid expressing the outer membrane porin MspA was present in these ESX-5 mutants to circumvent the essentiality of this system for growth of M. marinum. The mycP₁ knockout mutant did not show a growth defect in the presence of MspA. A mycP₁ knockout was created in M. marinum MUSA by allelic exchange using the phAE159 temperature-sensitive phage (33) and a method similar to that described for the creation of the mycP₅ and eccC₅ knockout by Ates et al. (4). The required construct was made by DNA amplification using primers MycP₁ LF, MycP₁ LR, MycP₁ RF, and MycP₁ RR (see Table S1 in the supplemental material) and the in-Fusion enzyme.

The chromosomal deletion was confirmed by PCR analysis and sequencing. The M. marinum E11 eccC₅ transposon mutant that was used in this study has been described previously by Stoop et al. (27).

**Cloning.** The mycP₁ and mycP₅ genes were amplified from M. marinum MUSA genomic DNA by PCR with anchored primers (EcoRI and

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**FIG 5** Model of the T7S membrane complex. (A) MycP₅ associates with the EccBCDE₅ (B/C/D/E) membrane-embedded complex, and, as a result, the T7S ESX-5 complex is stabilized and functional. IM, inner membrane; OM, outer membrane. (B) In the absence of MycP₅, the ESX-5 complex is less stable and, as a result, nonfunctional.
HindIII; see S1 Table in the supplemental material). Point mutations, the HA tag, and Twin-Strep-tag were introduced into mycP3 and mycP5 with nested primers (see Table S1). The generated constructs were additionally cloned as EcoRI-HindIII-digested fragments in pMV361 (34) or with PmlI and HindIII in the case of the Twin-Strep-tag. The C-terminal Twin-Strep-tag was introduced into eccCα by modifying the pMV-EccCα vector described by Ates et al. (4). The vector was digested with DraI and HindIII, and a linker consisting of two annealed oligonucleotides (see Table S1) was subsequently ligated to the digested vector. For the generation of pMV-EccBα-TwinStrep, the eccBα genes were amplified from M. marinum E11 genomic DNA with two consecutive PCRs. The first PCR amplified eccBα with an additional 100 to 200 bp on both sides of each gene, and the second PCR amplified eccBα and introduced a NsiI site in front of the gene (see Table S1). The PCR product was digested with NsiI and ligated into DraI- and NsiI-digested pMV-EccBα-Twin-Strep. MycP3mtnt, MycP1mtnt S334A, and EspBmtnt. E. coli expression plasmids were previously described by Wagner et al. (25). The mycP3mtnt::N239Y construct was amplified with anchored primers (Ndel and Xhol) using pET-21d-mycP1mtnt as a template, and the point mutation was introduced with nested primers (see Table S1). The construct was digested with Ndel and Xhol and ligated to Ndel- and Xhol-digested pet-28a. All plasmids were checked by sequencing of the relevant sections.

**Protein secretion and immunoblot analysis.** M. marinum strains were grown in TH9 liquid medium supplemented with ADC, 0.05% Tween 80, and appropriate antibiotics until mid-logarithmic phase, after which the cells were washed and inoculated in TH9 medium with 0.2% dextrose–0.05% Tween 80 at an optical density at 600 nm (OD600) of 0.4 and grown for another 16 h. The cells (Pellet) were spun down for 10 min at 6,000 × g, washed with phosphate-buffered saline (PBS), and resuspended in SDS loading buffer. Supernatants were passed through 0.45-μm-pore-size filter units, and proteins were precipitated with trichloroacetic acid (TCA) and resuspended in SDS loading buffer. Alternatively, the cells were resuspended in 0.5% Genapol X-080 and incubated for 1 h at room temperature. Samples were spun down and pellets were resuspended in SDS sample loading buffer (Genapol Pellet), while 5× SDS sample buffer was added to the supernatant containing Genapol X-080 (Genapol Supernatant). Proteins were separated on SDS-PAGE gels and transferred to a nitrocellulose membrane, and membranes were stained with anti-GroEL2 (C64); John Belise, NIH, Bethesda, MD, USA), antiPE-PGRS (31), anti-ESAT-6 (monoclonal antibody [MAB] Hyb76-8), anti-HA (HA.11; Covance), anti-EccB5 (2), anti-EccE5 (2), anti-EspE (Eric Brown; Genentech), anti-EccC5, anti-ESAT-6 (monoclonal antibody [MAb] Hyb76-8), and anti-GroEL2, or anti-EccE5 (2) antibodies. SDS solubilization buffer was added to the elution fractions, and samples were heated at 94°C, separated on a 10% SDS-PAGE gel, and visualized by Coomassie staining or transferred to a nitrocellulose membrane and stained with anti-EccB1, anti-EccB5, anti-EccC5 (2), anti-EccD3 (2), or anti-HA antibodies.

**Isolation of ESX-1 and ESX-5 membrane complexes and MycP3 pulldown.** Proteins were solubilized from isolated CE fractions as described above, with the addition of 0.3 mg/ml avidin (Sigma) after the DDM incubation. Solubilized proteins were incubated with Strep-Tactin beads for 30 min in a head-over-head manner, washed with 50 mM HEPES–KOH (pH 7.8)–150 mM KOAc–125 mM succrose–0.04% DDM, and eluted with 10 mM desthiobiotin, dissolved in the same buffer as was used for the washing. For the MycP3-Strep-tag purification, where stated, whole cells were treated with 1% formaldehyde and subsequently quenched with 100 mM glycine–10 mM NaHPO4 (pH 8.5). Proteins were solubilized from isolated CE fractions as described above, and the Strep-Tactin pulldown was performed as described above. SDS solubilization buffer was added to the elution fractions, and samples were heated at 94°C, separated on a 10% SDS-PAGE gel, and visualized by Coomassie staining or transferred to a nitrocellulose membrane and stained with anti-Strep-tag, anti-EccB1, anti-EccB5, anti-EccD3, or anti-EccE5 (2) antibodies. SDS solubilization buffer was added to the elution fractions, and samples were heated at 94°C, separated on a 12.5% SDS-PAGE gel, and visualized by Coomassie staining or transferred to a nitrocellulose membrane and stained with anti-Strep-tag, anti-EccB1, anti-EccB5, anti-EccD3, or anti-EccE5 (2) antibodies. LC-MS/MS. Peptides were separated by the use of an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, the Netherlands) equipped with a 20-cm-by-75-μm-inner-diameter (ID) fused-silica column custom packed with 3×120-Å repress Pur C18 aqua (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 μl/min on a 10-mm-by-100-μm-ID trap column packed with 5 μm 120-Å repress Pur C18 aqua using 2% buffer B (buffer A, 0.5% acetic acid–Milli-Q Water [MQ]; buffer B, 80% Acetonitrile [ACN]–0.5% acetic acid–MQ) and separated at 300 nl/min in a 10% to 40% buffer B gradient in 60 min (90 min, injection to injection). Eluting peptides were ionized at a potential of +2 kV into a Q Exactive mass spectrometer (Thermo, Fisher, Bremen, Germany). Intact masses were measured at a resolution of 70,000 (at m/z 200) in the Or-
bitrap using an automatic gain control (AGC) target value of $3 \times 10^6$ charges. The top 10 peptide signals (charge states 2+ and higher) were submitted to MS/MS in the high-cell-density (HCD) (higher-energy collision) cell (4-amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired at a resolution of 17,500 (at m/z 200) in the Orbitrap using an AGC target value of $2 \times 10^5$ charges and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

MS/MS spectra were searched against the Uniprot M. marinum complete proteome (ATCC BAA-535M) FASTA file (5,418 entries) using MaxQuant 1.4.1.2 (37). Enzyme specificity was set to trypsin, and up to two missed cleavages were allowed. Cysteine carboxamidomethylation (Cys; +57.021464 Da) was treated as a fixed modification and methionine oxidation (Met; +15.994915 Da) and N-terminal acetylation (N terminal; +42.010565 Da) as variable modifications. Peptide precursor ions were searched with a maximum mass deviation of 6.0 ppm and fragment ions with a maximum mass deviation of 20 ppm (default MaxQuant settings). Peptide and protein identifications were filtered at a false-discovery rate (FDR) of 1% using the decoy database strategy. Proteins that could not be differentiated based on MS/MS spectra alone were grouped into protein groups (default MaxQuant settings).

Proteins were quantified (in a label-free manner) by spectral counting, i.e., by determining the sum of all MS/MS spectra for each identified protein (38). For quantitative analysis across samples, spectral counts for identified proteins in a sample were normalized to the sum of spectral counts for that sample. This gives the spectral count contribution of a protein relative to the contribution of all spectral counts in the sample. For comparisons of different biological samples, these normalized spectral counts were used to calculate ratios. In this way, we were able to correct for loading differences between samples. Differential analysis of samples was performed using the beta-binominal test (39), which takes into account within- and between-sample variations, giving fold change values and associated P values for all identified proteins. Protein cluster analysis of the differentially expressed proteins was performed using hierarchical clustering in R. The protein abundances were normalized to zero mean and unit variance for each individual protein. Subsequently, the Euclidean distance measure was used for protein clustering.

**Accession number(s).** The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (40) partner repository with the data set identifier PXD003766.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01471-16/-/DCSupplemental.

- Figure S1, TIF file, 1.9 MB.
- Figure S2, TIF file, 0.8 MB.
- Figure S3, TIF file, 0.2 MB.
- Table S1, DOCX file, 0.01 MB.

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