Reconstitution of a minimal ESX-5 type VII secretion system suggests a role for PPE proteins in outer membrane transport of proteins

C. M. Bunduc[1,2,3,4], Y. Ding[1], C. Kuijl[5], T. C. Marlovits[2,3,4], W. Bitter[1,5], E.N.G. Houben[1#]

[1]Section Molecular Microbiology, Amsterdam Institute for Life and Environment (A-Life), Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

[2]Centre for Structural Systems Biology, Notkestraße 85, 22607 Hamburg, Germany

[3]Institute of Structural and Systems Biology, University Medical Center Hamburg-Eppendorf, Notkestraße 85, 22607 Hamburg, Germany

[4]German Electron Synchrotron Centre Notkestraße 85, 22607 Hamburg, Germany

[5]Department of Medical Microbiology and Infection Control, Amsterdam UMC, Amsterdam, The Netherlands

[#]Corresponding author. Vrije Universiteit Amsterdam, Section Molecular Microbiology, de Boelelaan 1108, 1081 HZ Amsterdam, the Netherlands. Tel.: +31 20 5983579; E-mail: e.n.g.houben@vu.nl

Keywords: ESX-5, mycobacterium, minimal system, secretion complex, type VII secretion

Abstract

Mycobacteria utilize type VII secretion systems (T7SSs) to secrete proteins across their highly hydrophobic and diderm cell envelope. Pathogenic mycobacteria have up to five different T7SSs, called ESX-1 to ESX-5, which are crucial for growth and virulence. Here, we use a functionally reconstituted ESX-5 system in the avirulent species *Mycobacterium smegmatis* that lacks ESX-5, to define the role of each *esx-5* gene in system functionality. By creating an array of gene deletions and assessing protein levels of components and membrane complex assembly, we observed that only the five components of the inner membrane complex are required for its assembly. However, in addition to these five core components, active secretion also depends on both the Esx and PE/PPE substrates. Tagging the PPE substrates followed by subcellular fractionation, surface labeling and membrane extraction showed that these proteins localize to the mycobacterial outer membrane. This indicates that they could play a role in secretion across this enigmatic outer barrier. These results provide a first full overview of the role of each *esx-5* gene in T7SS functionality.

Introduction

Gram-negative bacteria have evolved an exceptional variety of specialized secretion systems to mediate protein transport across their diderm cell envelope (Costa *et al*, 2015). Bacterial pathogens usually strictly depend on these systems to complete their infection cycle. Mycobacteria have an unusual diderm cell envelope with a distinctive outer membrane, also known as the mycomembrane, which is mainly composed of mycolic acids (Dulberger *et al*, 2020). These bacteria use a specific group of homologous specialized secretion systems, called type VII secretion systems, for a diverse array of functions, such as intracellular survival, immunomodulation, as well as uptake of nutrients and metabolites (Ates *et al*, 2015; Bunduc *et al*, 2020a; Pym *et al*, 2002; Siegrist *et al*, 2009; Simeone *et al*, 2012). Pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, encode up to five different T7SSs, named ESX-1 to ESX-5 (Bunduc *et al.*, 2020a). The importance of T7SS for pathogenicity is further evidenced by the lack of ESX-1 causing the attenuation of the currently used live vaccine strain *Mycobacterium bovis* BCG (Houben *et al*, 2012a; Pym *et al.*, 2002; Simeone *et al.*, 2012; van der Wel *et al*, 2007).

The mycobacterial *esx* loci contain five conserved T7SS membrane components, *i.e.* EccB, EccC, EccD and EccE (Ecc stands for ESX conserved component) and Mycosin or MycP. EccB is a single transmembrane domain (TMD) protein with a large periplasmic domain. EccC is a P-loop NTPase with four FtsK/SpoIIIE-like nucleotide binding domains (NBDs) and the motor protein of the complex, which has been shown to bind substrates (Champion *et al*, 2006; Rosenberg *et al*, 2015). EccD is the most hydrophobic component with eleven TMDs and EccE is anchored in the membrane via two TMDs. The four Ecc components have been shown to assemble into a large ~2 MDa machinery (Houben *et al*, 2012b) and several structures of (sub)complexes have been solved by electron microscopy (EM) (Beckham *et al*, 2017; Beckham *et al*, 2021; Famelis *et al*, 2019; Poweleit *et al*, 2019). The fifth membrane component MycP, a subtilisin-like protease with a single C-terminal TMD, interacts with and stabilizes the EccBCDE membrane complex (van Winden *et al*, 2020; van Winden *et al*, 2016). The recently solved cryo-EM structure of a full ESX-5 inner membrane complex of *M. tuberculosis* revealed the position of MycP within this complex, confirming the stabilizing role of MycP within the membrane assembly (Bunduc *et al*, 2021).

Most substrates that are secreted by mycobacterial T7SSs belong to three substrate families, *i.e.* the Esx, PE and PPE protein families. In pathogenic mycobacteria, especially the number of *pe* and *ppe* genes has expanded extensively, covering nearly 10% of the coding capacity (Cole *et al*, 1998). A specific feature of T7SS substrates is that they are secreted as folded heterodimers (Ates, 2020). Two different Esx proteins assemble into a secreted heterodimer, whereas PE proteins interact with specific

66 PPE proteins to form a structurally similar substrate pair. Dimerization is mediated by hydrophobic
67 interactions resulting in a conserved four helix bundle structure. The C-terminal flexible domain of one
68 of the secretion partner proteins additionally contains a YxxxD/E secretion signal that is important for
69 secretion (Daleke *et al*, 2012).

70 Mycobacterial T7SSs also have two cytosolic components, EccA and EspG. EspG is a PPE-
71 dedicated chaperone that binds to a hydrophobic patch on the so-called helical tip domain of these
72 proteins (Ekiert & Cox, 2014; Korotkova *et al*, 2014). Binding of EspG to PPE substrates is required to
73 keep PE/PPE pairs soluble in the cytosol (Ekiert & Cox, 2014; Korotkova *et al.*, 2014). EspG is additionally
74 involved in determining system-specific substrate recognition, as swapping the EspG-binding domain
75 between two PPE substrates resulted in rerouting of the ESX-1 substrate PPE68_1 to the ESX-5 system
76 in *Mycobacterium marinum* (Phan *et al*, 2017). EccA is an AAA+ ATPase that has been hypothesized to
77 disrupt the EspG/PPE interaction, leading to recycling of EspG in the cytosol, although the importance
78 of this ATPase for the secretion process remains controversial and could be dependent on growth
79 conditions (Ekiert & Cox, 2014; Phan *et al*, 2018).

80 Studying the functioning of T7SSs is impaired by the slow growth rate and virulence of
81 pathogenic mycobacteria. Certain systems, such as ESX-3 and ESX-5, are also essential for growth,
82 complicating genetic analyses (Ates *et al.*, 2015; Serafini *et al*, 2009; Siegrist *et al.*, 2009). Furthermore,
83 studying the potential role of specific substrates in the secretion process is hampered by the
84 redundancy between highly similar substrates (Ates, 2020; Gey van Pittius *et al*, 2006). *Mycobacterium*
85 *smegmatis*, an avirulent and fast-growing mycobacterium, is commonly used as a model for studying
86 mycobacterial physiology. This species possesses ESX-1, ESX-3 and ESX-4, but lacks the ESX-2 and ESX-
87 5 systems, which are exclusively found in slow-growing mycobacteria.

88 Here, we exploited an ESX-5 system of *Mycobacterium xenopi* that we have previously
89 functionally reconstituted in *M. smegmatis* (Beckham et al., 2017) to study the role of individual *esx-5*
90 genes in system functionality. We took advantage of the limited number of substrate genes present in
91 the introduced *esx-5* locus and defined the minimal number of genes necessary for successful assembly
92 of the ESX-5 membrane complex and ESX-5 mediated secretion.

93 ## Results

94

95 ### The inter-dependence of ESX-5 components for stable expression in *M. smegmatis*

96 To be able to efficiently dissect the individual roles of T7SS components, we used the ESX-5 system of
97 the slow-growing, moderately thermophilic and pathogenic mycobacterial species *M. xenopi* (Fig. 1A),
98 which we have previously functionally reconstituted in *M. smegmatis* (Beckham *et al.*, 2017; van
99 Winden *et al.*, 2020). As *M. smegmatis* does not have and never had a native ESX-5 system, the only
100 ESX-5 substrates are those encoded by the introduced *esx-5* locus, *i.e.* the Esx pair EsxM/EsxN and two
101 PE/PPE pairs that are specific for *M. xenopi* (Fig. 1A). This is in contrast to ESX-5 systems in natural hosts,
102 which secrete multiple Esx pairs and a large number of PE and PPE substrates, of which some show
103 redundancy (Abdallah *et al*, 2007; Ates, 2020). The limited number of substrates secreted by the
104 reconstituted system provides an excellent opportunity to analyze their individual roles in T7SS
105 functioning, bypassing any potential redundancy issues.

106 We exploited this non-essential reconstituted system to make single deletions of every *esx-5*
107 gene that encodes for system components, *i.e.* the *eccABCDE*, *mycP* and *espG* genes. In addition, we
108 created *esx-5* plasmids that were devoid of all *pe* and *ppe* genes (*Δpe/ppe*), lacking the two *esx* genes
109 (*ΔesxM/N*) or devoid of all genes that encode for substrates (*Δsubstrates*) (Fig. 1A). The *Δsubstrates* and
110 *Δpe/ppe* plasmids included the deletion of a non-conserved gene situated between the *pe/ppe* pairs,
111 *i.e. mxen9359,* which codes for a putative SAM-methyltransferase (Fig. 1A). Finally, we also created a
112 construct that only contained the five conserved membrane components (MC) (Fig 1A). These
113 constructs were used to assess the effect of each gene deletion on the protein levels of other
114 components, on the formation of the ESX-5 membrane complex and on ESX-5 mediated secretion.

115 First, we analyzed the impact of the various gene deletions on the expression of the other
116 system components by SDS-PAGE and immunoblotting, using specific antisera for the different ESX-5
117 components. We used isolated cell envelope fractions of *M. smegmatis* bearing the different *esx-5*
118 plasmids to detect membrane components (Fig. 1B) and whole cell lysates of the same strains to assess
119 protein levels of the cytosolic components (Fig. 1C). Deletion of the genes coding for the cytosolic
120 components $EspG_5$ and $EccA_5$ did not affect the protein levels of any of the five inner membrane
121 components, *i.e.* $EccB_5$, $EccC_5$ (strep tagged), $EccD_5$, $EccE_5$ and $MycP_5$ (Fig. 1B). Furthermore, in the
122 absence of $EccA_5$, the protein levels of $EspG_5$ were not affected and *vice versa* (Fig. 1C). Deleting the
123 membrane components $EccB_5$, $EccC_5$ or $MycP_5$ also did not affect protein levels of the four other
124 membrane components and the two cytosolic components. In contrast, the absence of $EccE_5$ seems to
125 affect protein levels of $EccD_5$ and also of $EccA_5$, although the latter could be attributed to polar effects
126 of the gene deletion, as *eccA*$_5$ lies downstream of *eccE*$_5$. Knocking out *eccD*$_5$ showed the most severe

127 effect on the protein levels of the other components. While $EccC_5$ and $EccE_5$ were undetectable by
128 immunoblotting in the absence of $EccD_5$, the protein levels of the membrane components $EccB_5$ and
129 $MycP_5$ were reduced. Strikingly, the two cytosolic components were also affected by the *eccD₅* deletion,
130 as $EspG_5$ could not be detected anymore and levels of $EccA_5$ were severely reduced. In conclusion,
131 deletion of the cytosolic components does not impact the protein levels of any of the other
132 components, while deleting *eccD₅* severely impacted the levels of all components.
133
134 **Only the five membrane components are required for full assembly of the ESX-5 membrane complex**
135 Next, we investigated which components play a role in the successful assembly of the ESX-5 inner
136 membrane complex. For this, we analyzed DDM-solubilized cell envelope fractions by blue native PAGE
137 (BN-PAGE), followed by immunoblotting using our anti-$EccB_5$ antibody or an antibody that recognized
138 the Strep-tag on $EccC_5$ (Fig 1D). Similar to previous observations (Beckham *et al.*, 2017), BN-PAGE
139 analysis of samples from *M. smegmatis* bearing the complete *esx-5* locus resulted in three main
140 complexes that reacted with the anti-$EccB_5$ antibody, the largest complex representing the full complex
141 of ~2 MDa and two subcomplexes of ~900 kDa and ~700 kDa. Staining with the anti-Strep antibody
142 showed a similar immunoblot pattern. Taking into consideration the influence of the detergent, lipids
143 and the Coomassie dye on the size estimation of membrane proteins by BN-PAGE (Wittig *et al*, 2010),
144 these three complexes could represent the full hexameric complex, the dimeric subcomplex and an
145 individual protomer, respectively. In the absence of the cytosolic components $EccA_5$ and $EspG_5$,
146 membrane complex assembly was not affected, as the BN-PAGE immunoblot pattern in their absence
147 was similar as for the WT construct (Fig. 1D). Similarly, none of the substrate deletion plasmids showed
148 differences in ESX-5 complexes, suggesting that substrates do not play a role in the assembly and/or
149 stability of the ESX-5 membrane complex. In line with this observation, a plasmid containing only the
150 five *esx-5* genes that code for the inner membrane components resulted in membrane complex
151 formation similar to the intact *esx-5* locus.
152 Deletion of the five membrane component genes, *i.e.* *eccB₅*, *eccC₅*, *eccD₅*, *eccE₅* and *mycP₅*,
153 showed varying effects on complex formation. In line with the observation that the *eccD₅* deletion
154 severely affected expression of the four other membrane components, no complex formation could be
155 observed in this condition. This suggests that $EccD_5$ is crucial for complex formation and/or stability,
156 which is in line with its central location within the protomers of the membrane complex (Fig. 1D). While
157 deletion of either *eccB₅*, *eccC₅* or *eccE₅* did not drastically affect the expression of the other membrane
158 components (Fig. 1B), the assembly of the membrane complex, as seen by BN-PAGE analysis, was
159 affected. Deletion of *eccB₅* affected formation of especially the full ~2 MDa complex, which is in line
160 with previous findings that $EccB_5$ is involved in complex multimerization (Bunduc *et al.*, 2021; Famelis

161 *et al.*, 2019). In the absence of the central ATPase $EccC_5$, which gates the potential secretion pore with
162 its TMDs, membrane complex formation was almost completely abolished, with this construct
163 exhibiting only a ~500 kDa subcomplex containing $EccB_5$, which was not observed with the WT
164 construct. Deleting *eccE*$_5$ showed a reduced amount of complexes, using both antibodies, which is in
165 line with our previous observation that a lower number of full complexes could be isolated under this
166 condition (Beckham *et al.*, 2017). In addition, the ~500 kDa complex, also present with the Δ*eccC*$_5$
167 construct, was observed in the absence of $EccE_5$ using the anti-$EccB_5$ but not the anti-Strep antibody.
168 Finally, while the *ΔmycP*$_5$ plasmid showed no major differences in the formation of the inner membrane
169 complex, the ~500 kDa $EccB_5$-containing complex was also observed here. These results were similar to
170 previous observations in *M. marinum*, where $MycP_1$ and $MycP_5$ of the ESX-1 and ESX-5 system,
171 respectively, have been reported to be involved in stabilizing its corresponding T7SS membrane
172 complex (van Winden et al., 2016).
173 Taken together, optimal assembly of the ESX-5 membrane complex requires only the five
174 membrane components.
175
176 *esx-5* locus-encoded PE/PPE proteins are required for secretion of Esx proteins
177 We previously observed that introduction of the plasmid that contains the complete *esx-5* locus of *M.*
178 *xenopi* in *M. smegmatis* resulted in the secretion of the locus-encoded ESX-5 substrate EsxN and also
179 of *M. marinum* PPE18, which was introduced by an additional plasmid containing the gene pair
180 *pe31/ppe18* (Beckham *et al.*, 2017). This shows that the reconstituted system is functional. We
181 therefore tested next the impact of the various gene deletions on the ability of this system to secrete
182 EsxN and PPE18, the latter by again introducing the plasmid containing the *pe31/ppe18* of *M. marinum*
183 (PPE18 containing a C-terminal HA-tag for detection) (Fig. 2A).
184 While both EsxN and PPE18-HA were efficiently secreted with the WT construct, they were also
185 secreted in the absence of *eccA*$_5$, albeit at a reduced level (Fig. 2A). Deletion of the other cytosolic
186 component *espG*$_5$ blocked the secretion of both PPE18-HA and EsxN (Fig. 2A). This critical role of $EspG_5$
187 for successful secretion of both the PE/PPE proteins and EsxM/EsxN is in line with previous findings
188 (Korotkova *et al.*, 2014). Additionally, PPE18-HA was not present in the bacterial pellet, suggesting that
189 $EspG_5$ is essential for the stability of this protein, as observed before for this and other PPE proteins
190 (Ekiert & Cox, 2014; Korotkova *et al.*, 2014; Phan *et al.*, 2017). In contrast, in the presence of only the
191 membrane components, which did not show secretion of PPE18-HA, this substrate could be detected
192 in the pellet fraction. The instability of PPE18 in the absence of $EspG_5$ can be rescued by deletion of
193 *eccA*$_5$ and/or the substrate-encoded *esx-5* genes. As all five membrane component deletions showed a
194 disruption in membrane complex assembly, it is no surprise that they also abolished the secretion of

195 both EsxN and PPE18-HA. Notably, deleting *eccD*$_5$ negatively affected also the protein levels of not only
196 PPE18-HA, which is in line with the absence of EspG$_5$ under this condition (Fig. 1C), but also of EsxN. In
197 the absence of EsxM/EsxN, secretion but not the stable expression of PPE18-HA was abrogated,
198 showing that this Esx heterodimer is required for secretion of the PE/PPE pair (Fig. 3A). Strikingly, in the
199 absence of PE/PPE proteins encoded by the *esx-5* locus, which did not affect expression of any ESX-5
200 components nor assembly of the ESX-5 membrane complex, secretion but not stable expression of both
201 EsxN and PPE18 was abolished (Fig. 2A). From this, we conclude that the PE/PPE pairs of the *esx-5* locus
202 are important for the secretion of the Esx substrates and at least one other PPE protein. This is in line
203 with a recent observation for ESX-1 in *M. marinum*, where secretion of EsxA is dependent on the co-
204 secretion of PPE68 encoded by the same gene cluster (Cronin *et al*, 2022; Damen *et al*, 2022).
205 To analyze the role of the individual *pe/ppe* gene pairs and of the putative methyltransferase
206 gene that is located between the two *pe/ppe* pairs, we created four complementation vectors
207 containing either the entire deleted region (whole; Fig. 1A), one of the two *pe/ppe* gene pairs with the
208 methyltransferase gene (*Δpe/ppe*$_1$ and *Δpe/ppe*$_2$) or only the two *pe/ppe* pairs without the
209 methyltransferase gene (*Δmethylt.*). EsxN secretion could be rescued by complementation with a
210 plasmid containing the entire deleted region (Fig. 2B), although the observed secretion levels were
211 reduced when compared to the levels with the WT construct. This reduction could be due to the fact
212 that both introduced plasmids contain the same origin of replication, thereby reducing individual
213 plasmid copy numbers and the overall protein levels. The same (reduced) secretion levels were
214 observed when we complemented with a plasmid that contains the entire deleted region without the
215 gene coding for the predicted methyltransferase, suggesting that this methyltransferase gene is not
216 required for secretion. Methyltransferases are not often associated with *esx-5* or other T7SS loci,
217 suggesting that the presence of this gene could be a coincidence. However, deletion of either of the
218 two *pe/ppe* pairs in the complementation construct abrogated secretion of EsxN completely. This shows
219 that both PE/PPE pairs are required for EsxN secretion. Notably, complementing the *Δpe/ppe* plasmid
220 with the PE31/PPE18-HA-encoding plasmid also did not restore secretion of EsxN (Fig. 2A). This shows
221 that although PPE18-HA is secreted in an ESX-5 dependent manner, it cannot take over the role of the
222 *esx-5* locus-encoded PE/PPE proteins in EsxN secretion, in line with a model in which Esx and PE/PPE
223 proteins are specifically paired for their co-secretion. The observation that both *esx-5*-encoded PE/PPE
224 proteins and EsxM/EsxN are required for PPE18-HA secretion shows that both substrate classes are
225 required for ESX-5 functionality.
226
227 PPE but not Esx substrates localize to the cell envelope fraction

228 While the interdependency of substrates for secretion can be explained by a previously proposed dual
229 substrate recognition event by the EccC ATPase, which is required for system activation (Bunduc *et al*,
230 2020b; Rosenberg *et al.*, 2015), substrates could additionally be dependent on each other beyond the
231 T7SS inner membrane complex. The dimensions of the core membrane complex dictate that it spans
232 only the inner membrane. How protein translocation takes place beyond the inner membrane,
233 especially across the mycobacterial outer membrane, is unknown, as there is no clear data on the
234 identity of the outer membrane channel or components. As our reconstituted ESX-5 system is
235 functional in *M. smegmatis*, the proteins that drive secretion over this second membrane are likely
236 encoded by the introduced *esx-5* cluster. Within the $esx\text{-}5_{Mxe}$ locus, the proteins that have thus far been
237 regarded as substrates are the only proteins that could mediate translocation through the outer
238 membrane, as all other components are localized either in the inner membrane or in the cytosol. In
239 addition, in recent years it has become clear that various PE/PPE proteins are involved in the uptake of
240 nutrients and localize to the cell envelope, with PPE51 as the main example (Korycka-Machala *et al*,
241 2020; Wang *et al*, 2020). To address whether the PPE substrates encoded by the *M. xenopi esx-5* locus
242 could be involved in outer membrane transport of proteins, we tagged the first and the second PPE
243 protein with a FLAG- and a Strep-tag, respectively, either at the N- or C-terminus. Additionally, to be
244 able to detect EsxN more efficiently, we added a C-terminal HA-tag to this substrate, which has
245 previously been shown not to interfere with the secretion of its ESX-1 equivalent EsxA (Damen *et al*,
246 2020). Subcellular fractionation and secretion analysis showed that a FLAG-tag at the C-terminus of the
247 first PPE protein, hereafter called PPE_1, partially affected secretion of EsxN, whereas placing the tag at
248 the N-terminus had no noticeable effect on EsxN secretion (Fig. EV 1A). The second PPE protein,
249 hereafter called PPE_2, could not be detected when tagged with a Strep-tag at the N-terminus and using
250 a Strep antibody, while placing the same tag to the C-terminus showed sufficient protein levels (Fig. EV
251 1A). With both constructs, EsxN was efficiently secreted to the culture supernatant (Fig. EV 1A),
252 suggesting that PPE_2 with the N-terminal tag is expressed, but that the tag is cleaved off. Finally, we
253 could also efficiently detect secreted EsxN-HA using the HA antibody.

254 With this information in hand, we made a final construct in which we removed the previously
255 introduced Strep-tag at the C-terminus of $EccC_5$ and modified the substrate genes, so that they encode
256 for PPE_1 with a FLAG-tag at the N-terminus, PPE_2 with a Strep-tag at the C-terminus and an HA-tag at
257 the C-terminus of EsxN. We first analyzed protein secretion by *M. smegmatis* carrying this construct
258 when grown in the presence or absence of Tween-80, a mild detergent used as anti-clumping agent for
259 mycobacteria grown in liquid culture (Fig. 3A). EsxN was present in the culture supernatant irrespective
260 of the presence of Tween-80. In contrast, both PPE proteins were found in the culture supernatant only
261 when cells were grown in the presence of the detergent. In the absence of Tween-80, these proteins

262 were found on the cell surface instead, as they could be extracted from the cell surface using the mild
263 detergent Genapol X-080 (Fig 3A). This suggests that the presence of Tween-80 triggers the release of
264 these proteins from the cell surface into the culture medium. Notably, when the gene encoding for the
265 central ATPase $EccC_5$ was deleted, neither PPE proteins were secreted anymore, showing that their
266 secretion is dependent on a functional ESX-5 system (Fig. EV 2).

267 Even though we could extract the PPE proteins, a substantial amount of both PPE proteins
268 remained cell-associated after growth with Tween-80 and/or after Genapol X-080 treatment. To
269 investigate the subcellular location of these subpopulations, *M. smegmatis* cells grown in the presence
270 of Tween-80 were lysed and fractionated into a soluble (cytosolic) and insoluble (cell envelope) fraction
271 (Fig. 3B). Intriguingly, both PPE proteins localized mainly to the cell envelope fraction. Furthermore,
272 flow cytometry analysis using anti-FLAG, -Strep and -HA antibodies revealed that both PPE proteins
273 could be efficiently detected on the cell surface of cells grown with Tween-80 (Fig. 3C, Fig. EV 1B). In
274 contrast, EsxN could not be detected via its C-terminal HA-tag, which is in line with the observation that
275 this substrate is mainly secreted into the culture medium. Interestingly, after treatment of the cells with
276 Genapol X-080, similar levels of surface labeling could be observed as compared to cells that were not
277 extracted with Genapol X-080 (Fig. 3C). This suggests that a significant portion of the cell-associated
278 population of the two PPE proteins is firmly attached to the cell surface. To characterize the cell
279 envelope association of these two PPE proteins further, we subjected isolated cell envelope fractions
280 from Genapol X-080-treated cells to solubilization with a range of detergents, some of which have been
281 used previously for the characterization of mycobacterial membrane proteins (Hoffmann *et al*, 2008;
282 Målen *et al*, 2010). While our inner membrane protein controls $EccC_5$ and FtsH could be extracted by
283 the nonionic detergents DDM, beta-octylglucoside and Triton X-100, and the anionic detergent SDS,
284 but not by Genapol X-080 or the zwitter ionic detergent CHAPSO, PPE_1 could be solubilized, partially or
285 fully, by all these detergents (Fig. 3C). In contrast, PPE_2 could not be solubilized by any of these
286 detergents, with the exception of SDS. Together, we conclude that the majority of the two *esx-5* locus-
287 encoded PPE proteins of *M. xenopi* localizes to the cell envelope of *M. smegmatis*, where they are
288 surface exposed. While a subpopulation of the PPE proteins associates with the cell surface in a Tween-
289 80/Genapol X-080 sensitive manner, a substantial amount is more firmly attached to the cell surface.
290 The observation that the second PPE protein cannot be extracted from the cell envelope with
291 detergents that are able to solubilize inner membrane proteins, a characteristic that is also observed
292 for known or predicted integral outer membrane proteins (Heinz & Niederweis, 2000; Speer *et al*,
293 2015), indicates that this protein is integrated into the mycobacterial outer membrane. This protein
294 could therefore potentially form a channel that mediates protein transport across the second
295 membrane of the mycobacterial cell envelope.

296 ## Discussion

297

298 By using a functionally reconstituted ESX-5 system in *M. smegmatis*, which naturally lacks this
299 system, we were able to study the role of each *esx-5* gene in component stability, assembly of the
300 membrane embedded machinery and system functionality. The obtained results are summarized in
301 Table 1.

302 Deletion of individual membrane components showed varying degrees of defects in membrane
303 complex assembly, while functionality of the ESX-5 system in all these mutants was completely
304 abolished. An *eccD*$_5$ mutant showed the most striking phenotype. Here, membrane complex assembly
305 was completely abolished, highlighting its role as a scaffold for the assembly of the inner membrane
306 complex. This mutant also exhibited highly affected protein levels, not only for the remaining
307 membrane components but also for cytosolic chaperones and substrates. The fact that the *esx-5* gene
308 cluster is introduced in *M. smegmatis* that lacks ESX-5 itself makes it unlikely that these reduced levels
309 are caused on a transcriptional level by a negative feedback loop, as has been described for the ESX-1
310 system (Abdallah *et al*, 2019; Bosserman *et al*, 2017; Chen *et al*, 2016). It suggests that the absence of
311 EccD$_5$ triggers a domino-like effect on the stability of the other proteins, starting with an unassembled
312 inner membrane machinery, which leads to degradation of the remaining membrane components and,
313 in the absence of the membrane complex, also of the components found in the cytosol, hereby
314 destabilizing the substrates.

315 Neither substrate nor chaperone mutants showed a defect in assembly of the membrane
316 complex and complex assembly required only the five conserved membrane components.
317 Nevertheless, conformational changes which cannot be accounted for by the methods used here could
318 still occur. While functionality was tightly connected with the successful assembly of the membrane
319 complex, the minimal functional T7SS ESX-5 unit requires the five membrane components, EspG$_5$, both
320 PE/PPE pairs that are encoded by the *esx-5* locus and an Esx heterodimer. EspG$_5$ proved to be critical
321 for successful secretion of both PE/PPE and EsxM/N proteins, similar to previous findings (Korotkova *et*
322 *al.*, 2014). The system without EccA$_5$ was still functional, although to a lower extent. EccA proteins have
323 been shown to be required for secretion in some but not all studies (Bottai *et al*, 2012; Joshi *et al*, 2012;
324 Phan *et al.*, 2018).

325 In addition, we showed that secretion of the Esx heterodimers by ESX-5 is dependent on the
326 locus encoded PE/PPE proteins. This is in line with a recent observation for ESX-1 in *Mycobacterium*
327 *marinum*, where secretion of EsxA is dependent on the co-secretion of PPE68 that is encoded by the
328 same gene cluster(Cronin *et al.*, 2022; Damen *et al.*, 2022). Notably, PPE68 was observed to be
329 degraded on the cell surface upon export. Since PE31/PPE18 was not able to complement secretion of

330 the Esx pair, our results are in line with a model in which Esx and PE/PPE proteins are specifically paired
331 for their co-secretion. The observation that both *esx-5* encoded PE/PPE proteins and EsxM/EsxN are
332 required for PPE18-HA secretion shows that both substrate classes are necessary for ESX-5
333 functionality.

334 Substrate interdependence for secretion, which has been observed also in other T7SSs (Cronin
335 *et al.*, 2022; Damen *et al.*, 2022), can be explained by previously proposed models in which the
336 membrane complex exhibits two separate recognition events, one between NBD3 of EccC and EsxB and
337 one mediated by the linker2 domain of EccC with PE/PPE proteins (Bunduc *et al.*, 2020b; Rosenberg *et*
338 *al.*, 2015). Substrate interdependency for secretion can also be a phenomenon taking place beyond the
339 inner membrane. As secretion of all three heterodimeric substrates across the full cell envelope is
340 dependent on the reconstituted ESX-5 system in our *M. smegmatis* model organism, all components
341 required for secretion across the cell envelope, including the outer membrane, are likely encoded on
342 the *esx-5*$_{xen}$ plasmid. Excluding inner membrane components and cytosolic chaperones leaves only the
343 substrates to be responsible for driving secretion over the outer membrane. Indeed, both PPE
344 substrates, but not EsxN, localize to the cell envelope fraction and are surface exposed, implying they
345 are embedded in the outer membrane. Furthermore, especially PPE_2, showed a phenotype consistent
346 with known mycobacterial outer membrane proteins, in that it cannot be readily solubilized by
347 detergents. Combined with their essential role in secretion of the Esx heterodimer and the exogenous
348 PE31/PPE18 pair, it suggests a new role for these specific PPE proteins in the transport of proteins
349 across the mycolic acid containing layer.

350 Based on the combined results, we propose two working models for the role of these *esx-5*-
351 encoded PPE proteins. In the first model, the roles of the two PPE proteins in secretion are different,
352 considering the differences in membrane association. Here, the first PPE protein could play a role in
353 activation of the inner membrane complex and co-secretion together with the EsxM/EsxN heterodimer,
354 similar as has been proposed for the ESX-1 dependent Esx and PE/PPE substrates (Cronin *et al.*, 2022;
355 Damen *et al.*, 2022) , while the second PPE protein could form the channel for protein translocation
356 over the outer membrane. In the second model, the two PPE proteins form the outer membrane
357 channel together, with PPE_2 creating the integral part and PPE_1 forming a peripheral portion of the
358 complex. As these PPE proteins are specific for *M. xenopi*, further studies are required to determine
359 whether the *esx-5*-associated PPE proteins of other mycobacterial species, such as *M. tuberculosis*,
360 have similar properties.

361 ## Materials and methods

362

363 ### Bacterial strains and growth conditions

364 *E. coli* Dh5α was grown in LB media supplemented with appropriate antibiotics at 37°C and 200 RPM.
365 *M. smegmatis* MC^2155 was grown in LB media supplemented with 0.5% Tween 80 (Merck) and
366 appropriate antibiotics at 37°C and 90 RPM. Antibiotics were used with the following concentrations:
367 hygromycin 50 mg/l, streptomycin 30 mg/ml.

368

369 ### Molecular cloning

370 Cloning was performed using *E. coli* Dh5α and restriction enzymes from New English Biolabs.
371 Polymerase chain reaction (PCR) products were amplified with IProof DNA polymerase from BioRad and
372 fused in multi-product reactions with InFusion HD from TakaraBio. For construction of plasmid
373 derivatives, genes were deleted, either completely if they were in a single operon or ~100-150 bp were
374 left at the C-terminus, if these were in an operon with other down- and/or upstream genes. Generally,
375 genes were deleted using the WT ESX-5_{Mxe} plasmid as template using the closest unique restriction sites
376 up- and downstream of the gene of interest and amplification of two or more PCR fragments with
377 compatible 18 bp ends, where the gene of interest was omitted. Because for different deletions the
378 same restriction sites were used, some primers were used for construction of multiple derivatives. For
379 a complete overview of restriction enzymes, primers, plasmids and cloning strategies see Table EV 1,
380 Table EV 2 and Table EV 3.

381

382 ### Protein secretion and immunoblot analysis

383 *M. smegmatis* MC^2155 with plasmids coding for ESX-5_{Mxe} WT or its derivatives were grown in LB liquid
384 medium with 0.05% Tween 80 and appropriate antibiotics at 37°C and 90 RPM until mid-log phase.
385 Cells were sub-cultured at an OD_{600} of 0.05 in LB liquid media under the same conditions for 12-16
386 hours until an OD_{600} of 0.8-1 was reached. Cultures were spun down, supernatants were passed through
387 0.2 µm filters and precipitated with trichloroacetic acid (TCA), resulting in culture supernatant fractions.
388 Cell pellets were broken via bead-beating with glass beads, resulting in whole cell lysates or bacterial
389 pellet fractions. SDS loading buffer was added to all samples, boiled and loaded on SDS-PAGE gels (10%
390 or 16% depending on the protein size), transferred to nitrocellulose membranes and stained with
391 appropriate antibodies. Antibodies against $EccA_5$, $EccB_5$, $EccD_5$, $EccE_5$, $EspG_5$, FtsH, $MycP_5$, EsxN
392 (Mtb9.9) and GroEL2 have been described previously (Houben *et al.*, 2012b); HA-, FLAG and Strep-
393 antibodies were purchased from Thermo Scientific (2-2.2.14), Sigma-Aldrich (F-1804) and Novusbio
394 (NBP2-41073).

395

396 Cell envelope isolation

397 *M smegmatis* MC^{2}155 with the various ESX-5$_{Mxe}$ plasmids were grown as above to an OD$_{600}$ of ~1.5.
398 Cells were washed in PBS, resuspended in buffer (50 mM Tris-HCl, 300 mM NaCl and 10% glycerol) and
399 broken by passing two times through a high-pressure homogenizer at 0.3 kbar (Stansted). Unbroken
400 cells were pelleted at 5000xg. Cell envelopes were separated from the soluble fraction by
401 ultracentrifugation at 150.000xg. After ultracentrifugation, pellets were washed and resuspended in
402 buffer and snap-frozen in liquid nitrogen. Where stated, after culture harvesting, cells were incubated
403 with mixing at room temperature with 0.5% Genapol X-80 for 30 minutes, to remove cell surface
404 proteins of bacteria. Subsequently, cells were centrifuged at 5000xg, washed one time with buffer and
405 fractionated as explained above.

406

407 BN-PAGE

408 For BN-PAGE analysis of membrane complexes, cell envelopes were solubilized with 0.25% DDM for
409 one hour at 4°C. Non-solubilized material was pelleted by centrifugation at 100.000xg for 20 minutes.
410 The resulting supernatant samples were run on 3-12% NativePage Bis-Tris Protein Gels (Invitrogen) with
411 addition of NativePage 5% G-250 Sample Additive (Invitrogen). Gels were blotted to PVDF membrane
412 and stained with appropriate antibodies.

413

414 Flow cytometry analysis

415 *M. smegmatis* expressing different plasmids was grown to an OD600 of 0.8 to 1.2 in LB with or without
416 Tween-80. Bacteria were pelleted, washed with PBS with 1% bovine serum albumin (BSA; Sigma), and
417 incubated for 2 h with antibodies recognizing the FLAG-tag (F-1804; Sigma-Aldrich), Strep-tag (NBP2-
418 41073; Novusbio) or influenza virus hemagglutinin tag (2-2.2.14; Thermo Scientific). After washing with
419 PBS with 1% BSA, bacteria were incubated with secondary goat anti-mouse IgG (A-21235;
420 ThermoFischer) or secondary goat anti-rabbit IgG (A-21244; ThermoFischer) conjugated to Alexa 647
421 antibodies for 30 min. After washing with PBS with 1% BSA, bacteria were analyzed by flow cytometry
422 (Attune NxT; ThermoFisher). As a control, bacteria were incubated only with secondary antibodies.

423

424 Acknowledgements

425 We thank Roy Ummels for technical help and advice. This work received funding by a VIDI grant
426 (864.12.006; to C.M.B. and E.N.G.H.) from the Netherlands Organization of Scientific Research. This
427 project has received funding from the European Union's Horizon 2020 research and innovation
428 programme under the Marie Sklodowska-Curie grant agreement no. 101030373 (to C.M.B.). This

project was supported by funds available to T.C.M. through the Behörde für Wissenschaft, Forschung und Gleichstellung of the city of Hamburg at the Institute of Structural and Systems Biology at the University Medical Center Hamburg–Eppendorf (UKE). The laboratory of T.C.M. is supported by DESY (German Electron Synchrotron Center).

Conflict of interest

The authors declare no conflict of interests.

References

Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W (2007) Type VII secretion--mycobacteria show the way. *Nat Rev Microbiol* 5: 883-891

Abdallah AM, Weerdenburg EM, Guan Q, Ummels R, Borggreve S, Adroub SA, Malas TB, Naeem R, Zhang H, Otto TD *et al* (2019) Integrated transcriptomic and proteomic analysis of pathogenic mycobacteria and their esx-1 mutants reveal secretion-dependent regulation of ESX-1 substrates and WhiB6 as a transcriptional regulator. *PLoS One* 14: e0211003

Ates LS (2020) New insights into the mycobacterial PE and PPE proteins provide a framework for future research. *Mol Microbiol* 113: 4-21

Ates LS, Ummels R, Commandeur S, van de Weerd R, Sparrius M, Weerdenburg E, Alber M, Kalscheuer R, Piersma SR, Abdallah AM *et al* (2015) Essential Role of the ESX-5 Secretion System in Outer Membrane Permeability of Pathogenic Mycobacteria. *PLoS Genet* 11: e1005190

Beckham KS, Ciccarelli L, Bunduc CM, Mertens HD, Ummels R, Lugmayr W, Mayr J, Rettel M, Savitski MM, Svergun DI *et al* (2017) Structure of the mycobacterial ESX-5 type VII secretion system membrane complex by single-particle analysis. *Nat Microbiol* 2: 17047

Beckham KSH, Ritter C, Chojnowski G, Ziemianowicz DS, Mullapudi E, Rettel M, Savitski MM, Mortensen SA, Kosinski J, Wilmanns M (2021) Structure of the mycobacterial ESX-5 type VII secretion system pore complex. *Sci Adv* 7

Bosserman RE, Nguyen TT, Sanchez KG, Chirakos AE, Ferrell MJ, Thompson CR, Champion MM, Abramovitch RB, Champion PA (2017) WhiB6 regulation of ESX-1 gene expression is controlled by a negative feedback loop in Mycobacterium marinum. *Proceedings of the National Academy of Sciences* 114: E10772-E10781

Bottai D, Di Luca M, Majlessi L, Frigui W, Simeone R, Sayes F, Bitter W, Brennan MJ, Leclerc C, Batoni G *et al* (2012) Disruption of the ESX-5 system of Mycobacterium tuberculosis causes loss of PPE protein secretion, reduction of cell wall integrity and strong attenuation. *Mol Microbiol* 83: 1195-1209

Bunduc CM, Bitter W, Houben ENG (2020a) Structure and Function of the Mycobacterial Type VII Secretion Systems. *Annu Rev Microbiol* 74: 315-335

Bunduc CM, Fahrenkamp D, Wald J, Ummels R, Bitter W, Houben ENG, Marlovits TC (2021) Structure and dynamics of a mycobacterial type VII secretion system. *Nature* 593: 445-448

Bunduc CM, Ummels R, Bitter W, Houben ENG (2020b) Species-specific secretion of ESX-5 type VII substrates is determined by the linker 2 of EccC5. *Mol Microbiol* 114: 66-76

Champion PA, Stanley SA, Champion MM, Brown EJ, Cox JS (2006) C-terminal signal sequence promotes virulence factor secretion in Mycobacterium tuberculosis. *Science* 313: 1632-1636

Chen Z, Hu Y, Cumming BM, Lu P, Feng L, Deng J, Steyn AJ, Chen S (2016) Mycobacterial WhiB6 Differentially Regulates ESX-1 and the Dos Regulon to Modulate Granuloma Formation and Virulence in Zebrafish. *Cell Rep* 16: 2512-2524

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, 3rd *et al* (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 393: 537-544

Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* 13: 343-359

Cronin RM, Ferrell MJ, Cahir CW, Champion MM, Champion PA (2022) Proteo-genetic analysis reveals clear hierarchy of ESX-1 secretion in Mycobacterium marinum. *Proc Natl Acad Sci U S A* 119: e2123100119

Daleke MH, Ummels R, Bawono P, Heringa J, Vandenbroucke-Grauls CM, Luirink J, Bitter W (2012) General secretion signal for the mycobacterial type VII secretion pathway. *Proc Natl Acad Sci U S A* 109: 11342-11347

485 Damen MPM, Meijers AS, Keizer EM, Piersma SR, Jiménez CR, Kuijl CP, Bitter W, Houben ENG
486 (2022) The ESX-1 Substrate PPE68 Has a Key Function in ESX-1-Mediated Secretion in Mycobacterium
487 marinum. *mBio* 13: e0281922
488 Damen MPM, Phan TH, Ummels R, Rubio-Canalejas A, Bitter W, Houben ENG (2020)
489 Modification of a PE/PPE substrate pair reroutes an Esx substrate pair from the mycobacterial ESX-1
490 type VII secretion system to the ESX-5 system. *J Biol Chem* 295: 5960-5969
491 Dulberger CL, Rubin EJ, Boutte CC (2020) The mycobacterial cell envelope - a moving target.
492 *Nat Rev Microbiol* 18: 47-59
493 Ekiert DC, Cox JS (2014) Structure of a PE-PPE-EspG complex from Mycobacterium tuberculosis
494 reveals molecular specificity of ESX protein secretion. *Proc Natl Acad Sci U S A* 111: 14758-14763
495 Famelis N, Rivera-Calzada A, Degliesposti G, Wingender M, Mietrach N, Skehel JM, Fernandez-
496 Leiro R, Bottcher B, Schlosser A, Llorca O *et al* (2019) Architecture of the mycobacterial type VII
497 secretion system. *Nature* 576: 321-325
498 Gey van Pittius NC, Sampson SL, Lee H, Kim Y, van Helden PD, Warren RM (2006) Evolution
499 and expansion of the Mycobacterium tuberculosis PE and PPE multigene families and their association
500 with the duplication of the ESAT-6 (esx) gene cluster regions. *BMC Evol Biol* 6: 95
501 Heinz C, Niederweis M (2000) Selective extraction and purification of a mycobacterial outer
502 membrane protein. *Anal Biochem* 285: 113-120
503 Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H (2008) Disclosure of the
504 mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid
505 bilayer structure. *Proc Natl Acad Sci U S A* 105: 3963-3967
506 Houben D, Demangel C, van Ingen J, Perez J, Baldeon L, Abdallah AM, Caleechurn L, Bottai D,
507 van Zon M, de Punder K *et al* (2012a) ESX-1-mediated translocation to the cytosol controls virulence
508 of mycobacteria. *Cell Microbiol* 14: 1287-1298
509 Houben EN, Bestebroer J, Ummels R, Wilson L, Piersma SR, Jimenez CR, Ottenhoff TH, Luirink
510 J, Bitter W (2012b) Composition of the type VII secretion system membrane complex. *Mol Microbiol*
511 86: 472-484
512 Joshi SA, Ball DA, Sun MG, Carlsson F, Watkins BY, Aggarwal N, McCracken JM, Huynh KK,
513 Brown EJ (2012) EccA1, a component of the Mycobacterium marinum ESX-1 protein virulence factor
514 secretion pathway, regulates mycolic acid lipid synthesis. *Chem Biol* 19: 372-380
515 Korotkova N, Freire D, Phan TH, Ummels R, Creekmore CC, Evans TJ, Wilmanns M, Bitter W,
516 Parret AH, Houben EN *et al* (2014) Structure of the Mycobacterium tuberculosis type VII secretion
517 system chaperone EspG5 in complex with PE25-PPE41 dimer. *Mol Microbiol* 94: 367-382
518 Korycka-Machala M, Pawelczyk J, Borowka P, Dziadek B, Brzostek A, Kawka M, Bekier A,
519 Rykowski S, Olejniczak AB, Strapagiel D *et al* (2020) PPE51 Is Involved in the Uptake of Disaccharides
520 by Mycobacterium tuberculosis. *Cells* 9
521 Målen H, Pathak S, Søfteland T, de Souza GA, Wiker HG (2010) Definition of novel cell envelope
522 associated proteins in Triton X-114 extracts of Mycobacterium tuberculosis H37Rv. *BMC Microbiol* 10:
523 132
524 Phan TH, Ummels R, Bitter W, Houben EN (2017) Identification of a substrate domain that
525 determines system specificity in mycobacterial type VII secretion systems. *Sci Rep* 7: 42704
526 Phan TH, van Leeuwen LM, Kuijl C, Ummels R, van Stempvoort G, Rubio-Canalejas A, Piersma
527 SR, Jimenez CR, van der Sar AM, Houben ENG *et al* (2018) EspH is a hypervirulence factor for
528 Mycobacterium marinum and essential for the secretion of the ESX-1 substrates EspE and EspF. *PLoS*
529 *Pathog* 14: e1007247
530 Poweleit N, Czudnochowski N, Nakagawa R, Trinidad DD, Murphy KC, Sassetti CM, Rosenberg
531 OS (2019) The structure of the endogenous ESX-3 secretion system. *Elife* 8: e52983
532 Pym AS, Brodin P, Brosch R, Huerre M, Cole ST (2002) Loss of RD1 contributed to the
533 attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti.
534 *Mol Microbiol* 46: 709-717

535 Rosenberg OS, Dovala D, Li X, Connolly L, Bendebury A, Finer-Moore J, Holton J, Cheng Y,
536 Stroud RM, Cox JS (2015) Substrates Control Multimerization and Activation of the Multi-Domain
537 ATPase Motor of Type VII Secretion. *Cell* 161: 501-512
538 Serafini A, Boldrin F, Palu G, Manganelli R (2009) Characterization of a Mycobacterium
539 tuberculosis ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J Bacteriol* 191: 6340-
540 6344
541 Siegrist MS, Unnikrishnan M, McConnell MJ, Borowsky M, Cheng TY, Siddiqi N, Fortune SM,
542 Moody DB, Rubin EJ (2009) Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition.
543 *Proc Natl Acad Sci U S A* 106: 18792-18797
544 Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, Enninga J (2012) Phagosomal
545 rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS Pathog* 8:
546 e1002507
547 Speer A, Sun J, Danilchanka O, Meikle V, Rowland JL, Walter K, Buck BR, Pavlenok M, Holscher
548 C, Ehrt S *et al* (2015) Surface hydrolysis of sphingomyelin by the outer membrane protein Rv0888
549 supports replication of *Mycobacterium tuberculosis* in macrophages. *Mol Microbiol* 97: 881-897
550 van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, Brenner M, Peters PJ
551 (2007) M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid
552 cells. *Cell* 129: 1287-1298
553 van Winden VJC, Bunduc CM, Ummels R, Bitter W, Houben ENG (2020) A Chimeric EccB-MycP
554 Fusion Protein is Functional and a Stable Component of the ESX-5 Type VII Secretion System
555 Membrane Complex. *J Mol Biol* 432: 1265-1278
556 van Winden VJC, Ummels R, Piersma SR, Jimenez CR, Korotkov KV, Bitter W, Houben EN (2016)
557 Mycosins Are Required for the Stabilization of the ESX-1 and ESX-5 Type VII Secretion Membrane
558 Complexes. *mBio* 7
559 Wang Q, Boshoff HIM, Harrison JR, Ray PC, Green SR, Wyatt PG, Barry CE, 3rd (2020) PE/PPE
560 proteins mediate nutrient transport across the outer membrane of Mycobacterium tuberculosis.
561 *Science* 367: 1147-1151
562 Wittig I, Beckhaus T, Wumaier Z, Karas M, Schagger H (2010) Mass estimation of native
563 proteins by blue native electrophoresis: principles and practical hints. *Mol Cell Proteomics* 9: 2149-
564 2161

565

566

567 Table 1. Overview of results.

Construct	Component/substrate protein levels				Complex formation	Secretion	
	Membrane	Cytosolic	EsxN	PPE18		EsxN	PPE18
WT	++	++	++	++	++	++	++
Δ*espG*$_5$	++	++	+	-	++	-	-
Δ*eccA*$_5$	++	++	+	+	++	+	+
Δ*eccB*$_5$	++	++	+	+	-	-	-
Δ*eccC*$_5$	+	++	+	+	-	-	-
Δ*eccD*$_5$	-	+/-	-	+/-	-	-	-
Δ*eccE*$_5$	+	+/-	+	+	+	-	-
Δ*mycP*$_5$	++	++	+	+	+	-	-
MC	++	n.a.	n.a.	+	++	n.a.	-
Δ*esxM/N*	++	++	n.a.	+	++	n.a.	-
Δ*pe/ppe*	++	++	+	+	++	-	-
Δ*substrates*	++	++	n.a.	+	++	n.a.	-

568 -, entirely affected; +/-, significantly affected; +, slightly affected; ++, not affected; n.a, not applicable

569

570 **Figure legends**

571

572 **Figure 1. Protein expression and membrane complex formation for ESX-5 mutants.**

573 (A) Genetic organization of the *esx-5* locus of *M. xenopi* RIVM700367 and several of its derivatives.
574 Depicted are the complete gene cluster, the cluster derivatives, in which more than one gene was
575 deleted, and the gene organization for the *pe*/*ppe* region complementation plasmid. (B) SDS-PAGE
576 and immunoblot analysis of cell envelope fractions of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid or
577 its derivatives using antibodies reactive against the four membrane components $EccB_5$, $EccD_5$, $EccE_5$
578 and $MycP_5$ or antibodies directed against a Strep-tag linked to the membrane component $EccC_5$.
579 Antibodies against the unrelated membrane protein FtsH were used as loading control. Notably,
580 $MycP_5$ is processed in one of the loops of its protease domain (van Winden *et al.*, 2016), resulting in
581 two immuno-reactive bands (arrow heads). The high hydrophobicity of $EccD_5$ compromises its
582 biochemical analysis, resulting in its variable detection by SDS-PAGE and immunoblotting. (C) SDS-
583 PAGE and immunoblot analysis of whole cell lysates of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid or
584 its derivatives using antibodies reactive against the cytosolic ESX-5 components $EccA_5$ and $EspG_5$.
585 Antibodies against GroEL2 were used as loading control. (D) Model of the ESX-5 system depicting all
586 components and substrates (left) and BN-PAGE and immunoblot analysis of DDM-solubilized cell
587 envelope fractions of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid or its derivatives using antibodies
588 against $EccB_5$ or the Strep-tag introduced on $EccC_5$ (right). Asterisks denote the ~500 kDa subcomplex
589 seen in the *$eccC_5$, $eccE_5$* and *$mycP_5$* mutants. MOM, mycobacterial outer membrane; IM, inner
590 membrane.

591

592 **Figure 2. Secretion analysis of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid or its derivatives.**

593 (A) SDS-PAGE and immunoblot analysis of secreted fractions (culture supernatants) and whole cell
594 lysates (bacterial pellets) using antibodies against EsxN. In addition, an HA-antibody was used to
595 detect PPE18-HA that was encoded together with PE31, its partner protein, on a separate plasmid.
596 Antibodies against GroEL2 were used as lysis and loading control for the whole cell lysates and
597 antibodies against Apa were used as loading control for the secreted fraction. (B) Secretion analysis
598 of *M. smegmatis* carrying the ESX-5_{Mxe} Δ*pe/ppe* plasmid and various *pe/ppe* complementation vectors.
599 The used bacterial fractions and antibodies are the same as under A.

600

601 **Figure 3. Subcellular localization of the tagged PPE substrates of the *esx-5_{Mxe}* locus expressed in *M.*
602 *smegmatis***

603 (A) Secretion analysis of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid expressing tagged substrates.
604 SDS-PAGE and immunoblot analysis of secreted fractions (culture supernatants) and whole cell lysates
605 (bacterial pellets) of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid encoding the first PPE substrate
606 (PPE_1) with a FLAG-tag at the N-terminus, the second PPE protein (PPE_2) with a Strep-tag at the C-
607 terminus and EsxN with an HA-tag at the C-terminus. Antibodies against GroEL2 were used as lysis
608 control. Cells were grown in the presence or absence of Tween-80, after which they were harvested
609 and half was treated with Genapol X-080 to extract surface proteins. P, bacterial pellet; S, culture
610 supernatant; GP, Genapol pellet; GS, Genapol supernatant. Background bands are marked with
611 asterisks. (B) Cells expressing the *M. xenopi esx-5* locus encoding substrates with tags were grown in
612 the presence of 0.05% Tween 80, after which cells were fractionated into total (T), cytosol (Cyt) and
613 cell envelope (CE) fractions. Fractions were loaded in equal amounts. Antibodies against FtsH (inner
614 membrane protein) and GroEL2 (cytosolic protein) were used as fractionation control. (C) Surface
615 exposure of substrates on whole cells was measured by flow cytometry of cells grown in the presence
616 of Tween-80 without (top) or with (bottom) surface protein extraction with Genapol X-080 after
617 culture harvesting. (D) SDS-PAGE and immunoblot or Coomassie brilliant blue (CBB) stain analysis of
618 cell envelope fractions isolated from cells treated with Genapol X-080 and solubilized with the
619 mentioned detergents. Concentrations for the detergents were: 0.25% DDM, 1% n-Octyl-β-D-
620 Glucopyranoside, 2% Triton X-100, 2% SDS, 0.5% Genapol X-080 and 5% CHAPSO. Fractions were
621 loaded in equal amounts. Antibodies against $EccB_5$ and FtsH were used as inner membrane controls.
622 S, soluble fraction; P, non-soluble fraction.
623
624 **Expanded View Figure 1. Subcellular fractionation of *M. smegmatis* carrying the *esx-5$_{Mxe}$* plasmid**
625 **expressing tagged ESX-5 substrates.**
626 (A) Cells expressing the *M. xenopi esx-5* locus encoding substrates with tags at the N or C- termini
627 were grown in the presence of 0.05% Tween 80, after which supernatants (S) were separated from
628 bacterial cells. The cells were subsequently fractionated into total (T), cytosol (Cyt) and cell envelope
629 (CE) fractions. Fractions were loaded in a ratio of 2:1:1:1 for S:T:Cyt:CE. Antibody against FtsH (inner
630 membrane protein) was used as fractionation control. (B) Surface exposure of substrates on whole
631 cells was measured by flow cytometry of cells grown in the presence of Tween-80.
632
633 **Expanded View Figure 2. Secretion analysis of the *esx-5*-encoded PPE proteins by *M. smegmatis***
634 **carrying the *esx-5$_{Mxe}$* Δ*eccC$_5$* plasmid.** SDS-PAGE and immunoblot analysis of secreted fractions (S) and
635 whole cell lysates (P) of *M. smegmatis* carrying the ESX-5_{Mxe} plasmid encoding the first PPE substrate
636 (PPE_1) with a FLAG-tag at the N-terminus, the second PPE protein (PPE_2) with a Strep-tag at the C-

637 terminus and EsxN with a HA-tag at the C-terminus and the same plasmid with *eccC*$_5$ deleted.
638 Antibodies against GroEL2 were used as lysis control. Cells were grown in the presence of Tween-80.
639
640 **Expanded View Table 1.** List of plasmids used in this study
641
642 **Expanded View Table 2.** Primers used in this study for molecular cloning
643
644 **Expanded View Table 3.** Overview of molecular cloning strategies for generating the plasmids shown
645 in Table EV1 and using primers shown in Table EV2.
646

Figure 1

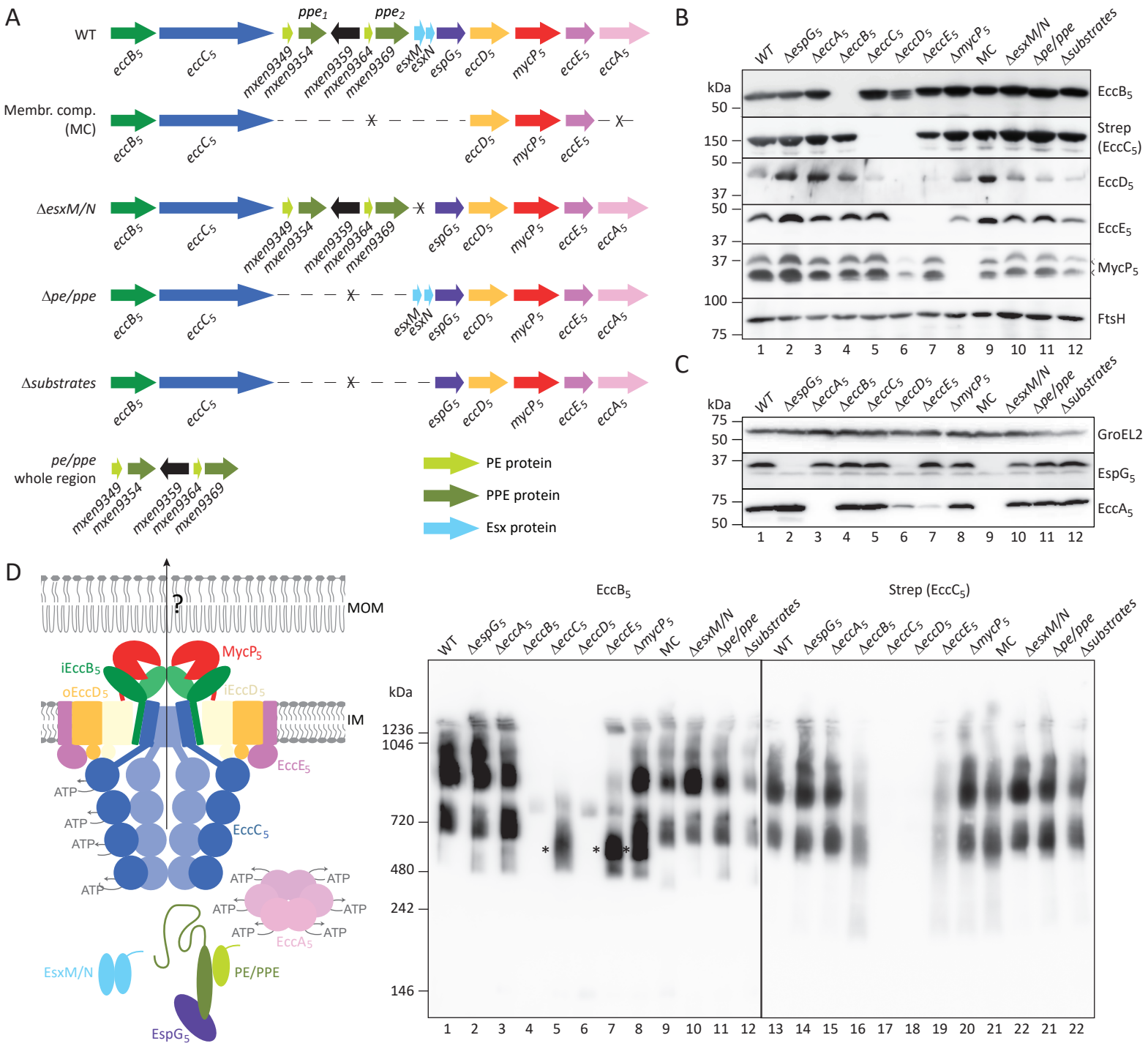
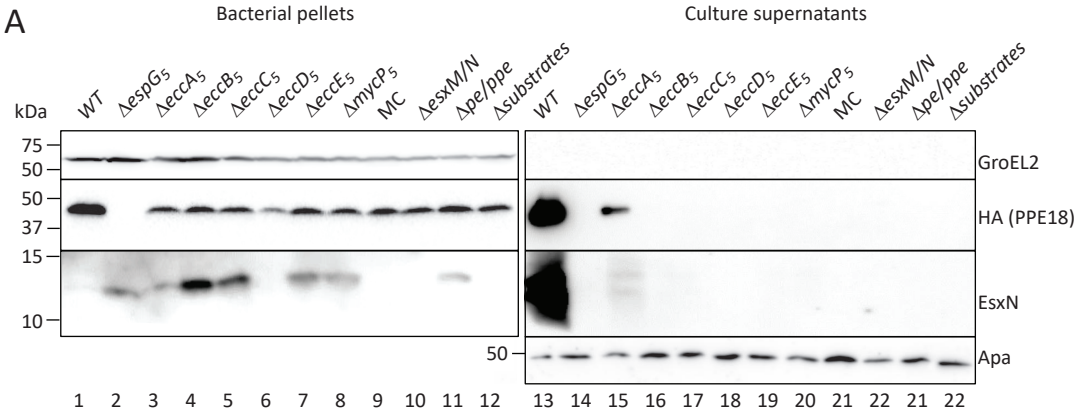

A
WT
Membr. comp. (MC)
ΔesxM/N
Δpe/ppe
Δsubstrates
pe/ppe whole region
eccB5
eccC5
mxen9349
mxen9354
mxen9359
mxen9364
mxen9369
ppe1
ppe2
esxM
esxN
espG5
eccD5
mycP5
eccE5
eccA5
PE protein
PPE protein
Esx protein
B
WT
ΔespG5
ΔeccA5
ΔeccB5
ΔeccC5
ΔeccD5
ΔeccE5
ΔmycP5
MC
ΔesxM/N
Δpe/ppe
Δsubstrates
kDa
EccB5
Strep (EccC5)
EccD5
EccE5
MycP5
FtsH
C
GroEL2
EspG5
EccA5
D
MOM
IM
?
MycP5
iEccB5
oEccD5
iEccD5
EccE5
EccC5
EccA5
ATP
EsxM/N
PE/PPE
EspG5
EccB5
Strep (EccC5)
1236
1046
720
480
242
146

Figure 2


A
Bacterial pellets
Culture supernatants
kDa
WT
ΔespG5
ΔeccA5
ΔeccB5
ΔeccC5
ΔeccD5
ΔeccE5
ΔmycP5
MC
ΔesxM/N
Δpe/ppe
Δsubstrates
75
50
37
15
10
GroEL2
HA (PPE18)
EsxN
Apa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 21 22

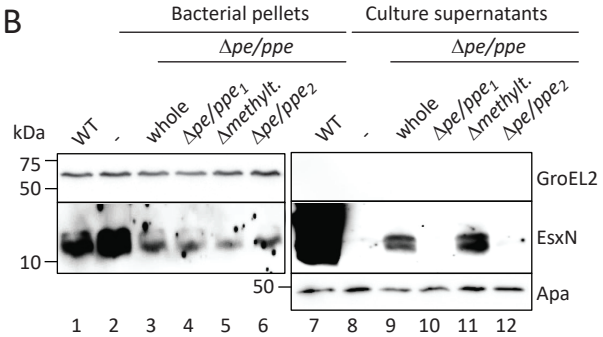
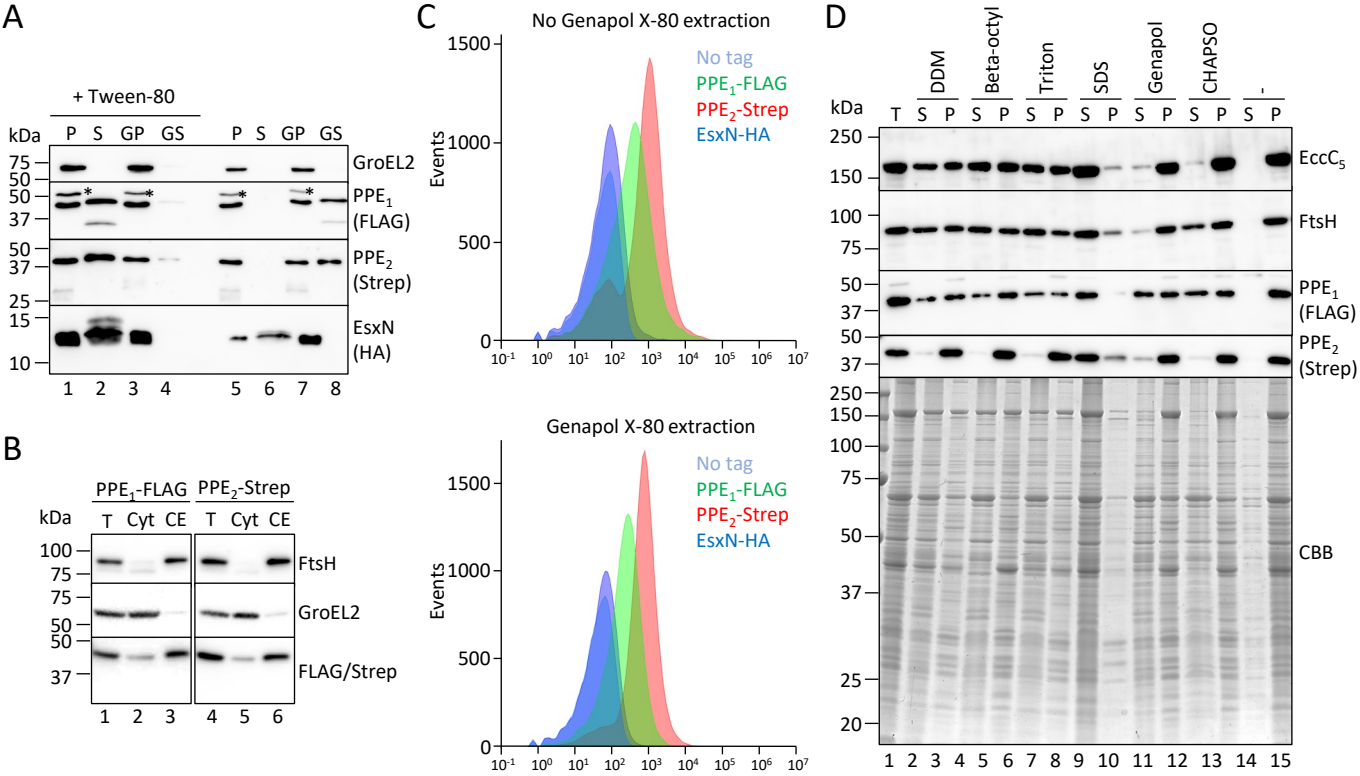
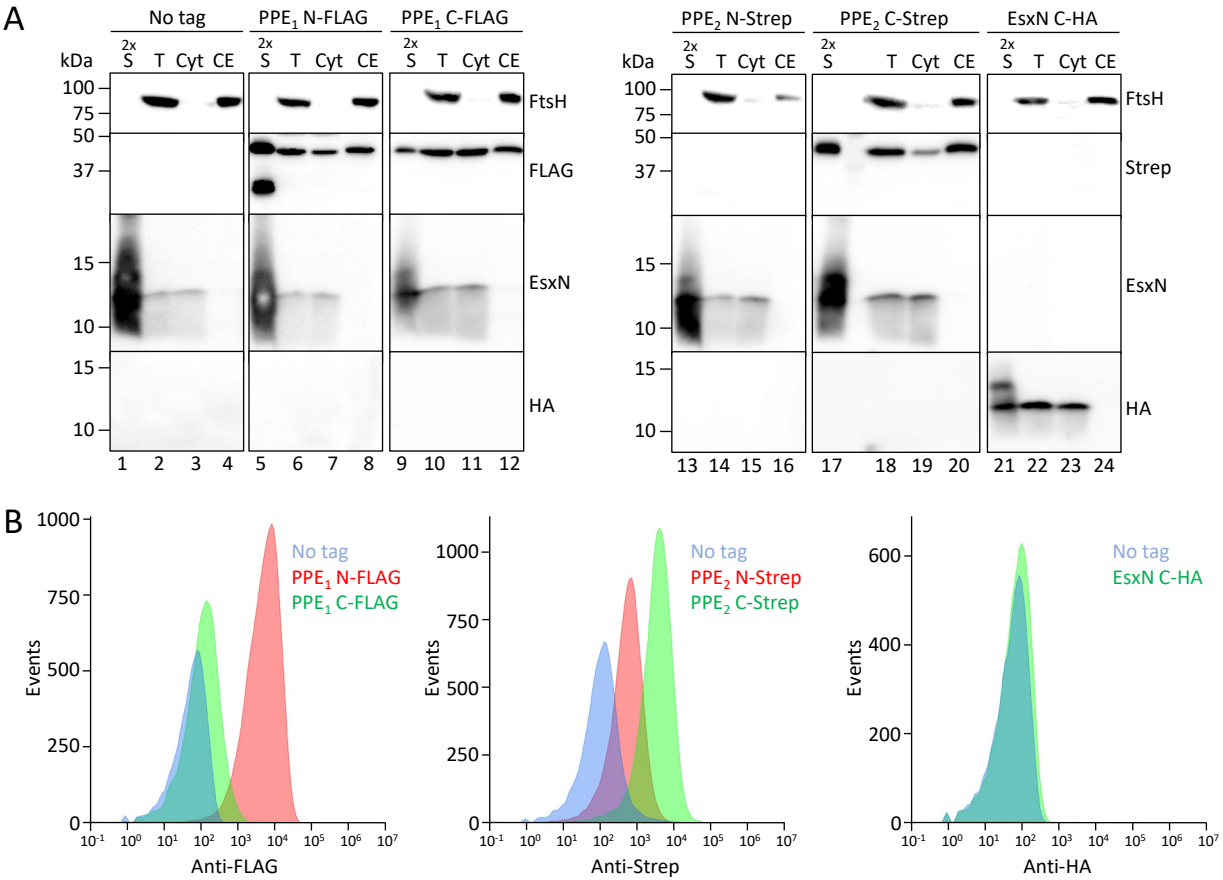

B
Bacterial pellets
Culture supernatants
Δpe/ppe
Δpe/ppe
kDa
WT
-
whole
Δpe/ppe1
Δmethylt.
Δpe/ppe2
75
50
10
GroEL2
EsxN
Apa
1 2 3 4 5 6 7 8 9 10 11 12

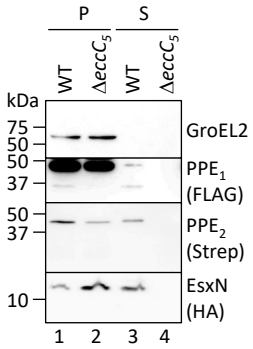
Figure 3


A
+ Tween-80
kDa
P S GP GS P S GP GS
75
50
50
37
50
37
25
15
10
GroEL2
PPE1 (FLAG)
PPE2 (Strep)
EsxN (HA)
1 2 3 4 5 6 7 8
B
PPE1-FLAG PPE2-Strep
kDa
T Cyt CE T Cyt CE
100
75
75
50
50
37
FtsH
GroEL2
FLAG/Strep
1 2 3 4 5 6
C
No Genapol X-80 extraction
Events
No tag
PPE1-FLAG
PPE2-Strep
EsxN-HA
Genapol X-80 extraction
D
DDM
Beta-octyl
Triton
SDS
Genapol
CHAPSO
-
kDa
T S P S P S P S P S P S P S P
250
150
100
75
50
37
25
20
EccC5
FtsH
PPE1 (FLAG)
PPE2 (Strep)
CBB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Expanded View Figure 1



Expanded View Figure 2



Expanded View Table 1: List of plasmids used in this study.

Nr.	Construct	Source
1.	pMV *esx-5*$_{Mxe}$	Beckham *et al*., 2017
2.	pMV *esx-5*$_{Mxe}$ Δ*eccB*$_5$	this study
3.	pMV *esx-5*$_{Mxe}$ Δ*eccC*$_5$	this study
4.	pMV *esx-5*$_{Mxe}$ Δ*pe/ppe*	this study
5.	pMV *esx-5*$_{Mxe}$ Δ*esxM/N*	this study
6.	pMV *esx-5*$_{Mxe}$ Δ*substrates*	this study
7.	pMV *esx-5*$_{Mxe}$ Δ*espG*$_5$	this study
8.	pMV *esx-5*$_{Mxe}$ Δ*eccD*$_5$	this study
9.	pMV *esx-5*$_{Mxe}$ Δ*mycP*$_5$	van Winden *et al*., 2020
10.	pMV *esx-5*$_{Mxe}$ Δ*eccE*$_5$	this study
11.	pMV *esx-5*$_{Mxe}$ Δ*eccA*$_5$	this study
12.	pMV *esx-5*$_{Mxe}$ membrane components (MC)	this study
13.	pSMT3 *pe/ppe* whole	this study
14.	pSMT3 *pe/ppe* Δ*pe/ppe*$_1$	this study
15.	pSMT3 *pe/ppe* Δmethyltransferase	this study
16.	pSMT3 *pe/ppe* Δ*pe/ppe*$_2$	this study
17.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term	this study
18.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG C-term	this study
19.	pMV *esx-5*$_{Mxe}$ *ppe*$_2$ Strep N-term	this study
20.	pMV *esx-5*$_{Mxe}$ *ppe*$_2$ Strep C-term	this study
21.	pMV *esx-5*$_{Mxe}$ *esxN-HA*	this study
22.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term, *ppe*$_2$ Strep C-term, *esxN-HA*, No Strep on *eccC*$_5$	this study
23.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term, *ppe*$_2$ Strep C-term, *esxN-HA*, Δ*eccC*$_5$	this study

Expanded View Table 2: List of primers used in this study.

Nr.	Primer name	Primer sequence 3' -5'
1.	ESX-5 xen del.EccB 1 FW	TGTTTGCTTGTCGGACACCT
2.	ESX-5 xen del.EccB 1 RV	CTATTTGAGTCACGATCCCCGTTC
3.	ESX-5 xen del.EccB 2 FW	GATCGTGACTCAAATAGGCACCATGGGTGGCACTG
4.	ESX-5 xen del.EccB 2 RV	TCCCATTGCGGGTCTTCGAT
5.	ESX-5 xen del.EccC FW	GTACGCGAAGAACCACTTAATTAATGC
6.	ESX-5 xen del.EccC RV	GAGGTGCCTGCCTTAAGTCACTTCGGTACCGCCAATTC
7.	Del EspG xen FW	GGCGGGGGCTTCTCTAGATG
8.	Del EsxM/N xen 1 rv	TGCGGTTGGTTGTTACTGAATATCTCCTTAGCTCGAAATCGGCTG
9.	Del EsxM/N xen 2 fw	TAACAACCAACCGCAGGCGC
10.	Del EsxM/N xen 2 rv	CATCTCGACCGAATACGTAAGTGAAAG
11.	Xen ESX-5 del sub. INF. FW	CGAGAAGTAAAGCTCTTAAGTAACAACCAACCGCAGGCGCG
12.	Del EspG xen FW	GGCGGGGGCTTCTCTAGATG
13.	Del EspG xen RV	CATCTCGACCGAATACgtaGGCTGCTCCCATGTTGGCGTG
14.	Del EccE clean 1 FW	GATCCTGCGCGGCCGCTCAT
15.	Del EccE clean 1 RV	CAGCCGTGGGCGTCGTCACGTCATGGCTGCCTCCCTTGTCG
16.	Del EccE clean 2 FW	CGTGACGACGCCCACGGCTGGTG
17.	Del EccE clean 2 RV	CGTCGACATCGATAAGCTTTGG
18.	Del EccA xen FW	GCCATCTGCCGGACGCGTG
19.	Del EccA xen RV	GTCGACATCGATAAGCTTCATCGGGCCGCCGCCGGG
20.	Xen whole PE/PPE inf FW	GAGGAATCACGCTAGCGATGTCGTTCGTGACC
21.	Xen whole PE/PPE inf RV	AGATATCCATGGATCCTAACCGGCGTAGACC
22.	Xen tag substr 1 FW	CGAGAAGTAAAGCTCTTAAGGCAGGCAC
23.	Xen 1st PPE flag C 1 RV	TGTCGTCATCGTCTTTGTAGTCGCCGGCGAACGGTGGGCGTG
24.	Xen 1st PPE Flag C 2 FW	ACAAAGACGATGACGACAAGTAGCAACACGCGAAGCGCCG
25.	Xen tag substr 2 RV	TGCATCTCGACCGAATACGTAAGTGAAA
26.	Xen 2nd PPE Strep C 1 RV	TTCAAATTGGGGATGCGACCATGATCCTCCACCGGCGTAGACCGGGCG
27.	Xen 2nd PPE Strep C 2 FW	TCGCATCCCCAATTTGAAAAATAGGAAAGGGGGCCAGCCG
28.	Xen EsxN-HA 1 RV	TAATCAGGAACATCATACGGATAGGCCCAGCTGGAGCCGAC
29.	Xen EsxN-HA 2 FW	CGTATGATGTTCCTGATTATGCTTAACAACCAACCGCAGGCGC
30.	Xen 1st PPE flag Nterm 1 RV	CGAACCTCCCGAACCTCCCTTGTCGTCATCGTCTTTGTAGTCCATTAGTACTCTCCTTAGCCCGCCG
31.	Xen 1st PPE Flag Nterm 2 FW	GGAGGTTCGGGAGGTTCGGTGCTGGACTTCGGGGCATTTC
32.	Xen 2nd PPE Strep Nterm 1 RV	CGAACCTCCCGAACCTCCTTTTTCAAATTGGGGATGCGACCACATCTAGAGAAGCCCCCGCC
33.	Xen 2nd PPE Strep Nterm 2 FW	GGAGGTTCGGGAGGTTCGTTGGATTTCGGGCTGTTTCCG
34.	Rem Str EccC5xen FW	CCGACCTGCGCCGGTTTAAATAGCTTAAGGCAGGCACCTCTTGAG
35.	Rem Str EccC5xen RV	CTCAAGAGGTGCCTGCCTTAAGCTATTTAAACCGGCGCAGGTCGG

Expanded View Table 3: Overview of molecular cloning strategies for generating the plasmids shown in Expanded View Table 1 and using primers shown in Expanded View Table 2.

Nr.	Construct	Starting backbone	Restriction sites used to cut the backbone	Primers used	PCR product size	Comments
2.	pMV *esx-5*$_{Mxe}$ Δ*eccB*$_5$	pMV *esx-5*$_{Mxe}$	EcoRV, SexAI	1, 2 3, 4	330 bp 1140 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
3.	pMV *esx-5*$_{Mxe}$ Δ*eccC*$_5$	pMV *esx-5*$_{Mxe}$	PacI, AflII	5, 6	2174 bp	Restricted backbone and the PCR product were ligated with InFusion HD.
4.	pMV *esx-5*$_{Mxe}$ Δ*pe/ppe*	pMV *esx-5*$_{Mxe}$	AflII, XbaI			Restriction removes *pe/ppe* genes. Resulting backbone was recircularized.
5.	pMV *esx-5*$_{Mxe}$ Δ*esxM/N*	pMV *esx-5*$_{Mxe}$	Xbai, SnaBI	7, 8 9, 10	1356 bp 1061 bp	Restricted backbone and the two PCR products were ligated in InFusion HD.
6.	pMV *esx-5*$_{Mxe}$ Δ*substr.*	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	11, 10	1082 bp	Restricted backbone and the PCR product were ligated with InFusion HD.
7.	pMV *esx-5*$_{Mxe}$ Δ*espG*$_5$	pMV *esx-5*$_{Mxe}$	XbaI, SnaBI	12, 13	2053 bp	Restricted backbone and the PCR product were ligated with InFusion HD.
8.	pMV ESX-5xen Δ*eccD*$_5$	pMV *esx-5*$_{Mxe}$	SnaBI, NdeI			Restriction removes *eccD*$_5$. Resulting backbone was recircularized.
10.	pMV ESX-5xen Δ*eccE*$_5$	pMV *esx-5*$_{Mxe}$	NdeI, HindIII	14, 15 16, 17	2068 bp 2607 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
11.	pMV ESX-5xen ΔEccA5	pMV *esx-5*$_{Mxe}$	MluI, HindIII	18, 19	1129 bp	Restricted backbone and the PCR product were ligated with InFusion HD.
12.	pMV ESX-5xen MC	pMV *esx-5*$_{Mxe}$ ΔEccA5	AflII, SnaBI			Restriction removes all substrate genes and *espG*$_5$. Resulting backbone was recircularized.
13.	pSMT3 *pe/ppe* whole	pSMT3 empty		20, 21	4583 bp	Backbone and PCR product were ligated with InfusionHD.
14.	pSMT3 pe/ppe Δ*pe/ppe*$_1$	pSMT3 *pe/ppe* whole	NheI			Restriction removes 1st *pe/ppe* genes. Resulting backbone was recircularized.
15.	pSMT3 *pe/ppe*Δmethyltransf.	pSMT3 *pe/ppe* whole	NheI, BamHI			Partial restriction to delete methyltransferase. Resulting backbone was recircularized.
16.	pSMT3 *pe/ppe* Δ*pe/ppe*$_2$	pSMT3 *pe/ppe* whole	BamHI			Restriction remove 2nd *pe/ppe* genes. Resulting backbone was recircularized.
17.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	22, 30 31, 25	688 bp 6035 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
18.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG C-term	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	22, 23 24, 25	1934 bp 4769 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
19.	pMV *esx-5*$_{Mxe}$ *ppe*$_2$ Strep N-term	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	22, 32 33, 25	3716 bp 3005 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
20.	pMV *esx-5*$_{Mxe}$ *ppe*$_2$ Strep C-term	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	22, 26 27, 25	4910 bp 1802 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
21.	pMV *esx-5*$_{Mxe}$ *esxN-HA*	pMV *esx-5*$_{Mxe}$	AflII, SnaBI	22, 28 29, 25	5621 bp 1802 bp	Restricted backbone and the two PCR products were ligated with InFusion HD.
22.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term, *ppe*$_2$ Strep C-term, *esxN-HA*, no Strep on *eccC*$_5$.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ Flag N-term pMV *esx-5*$_{Mxe}$ *esxN-HA* Intermediate plasmid	AflII, SnaBI AflII, SnaBI DraI, AflII	22, 26 27, 25 34, 35	4910 bp 1802 bp	Restricted backbone and the two PCR products were ligated with InFusion HD into an intermediate plasmid. Restricted intermediate plasmid was religated with InFusion HD with the overlapping primers to swap the TwinStrep at the C-terminus of *eccC*$_5$ with a stop codon.
23.	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term, *ppe*$_2$ Strep C-term, *esxN-HA*, Δ*eccC*$_5$	pMV *esx-5*$_{Mxe}$ *ppe*$_1$ FLAG N-term, *ppe*$_2$ Strep C-term, *esxN-HA*, No Strep on *eccC*$_5$.	PacI, AflII	5, 6	2174 bp	Restricted backbone and the PCR product were ligated with InFusion HD.