# Reconstitution of a minimal ESX-5 type VII secretion systems uncovers the essential role of the PPE proteins in secretion

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

## 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 gene of the *esx-5* locus in ESX-5 functionality. By creating an array of gene deletions and assessing ESX-5 component protein levels and membrane complex assembly, we observed that only the five components of the inner membrane complex are required for the assembly of this complex. However, in addition to these five core members, active secretion also depends on both the Esx proteins and the PE/PPE substrates. Tagging the PPE substrates followed by subcellular localization, membrane extraction and surface labeling showed that these proteins localize to the cell envelope, indicating that they could play a role in secretion across the mycobacterial outer membrane. These results provide a first blueprint on the role of each component within mycobacterial T7SSs.

## 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 mainly composed of mycolic acids, also known as the mycomembrane (Dulberger *et al*, 2020). These bacteria use a specific group of homologous specialized secretion systems, also called the 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 for 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 dictating the attenuation of the live vaccine strain, *Mycobacterium bovis BCG* (Houben *et al*, 2012a; Simeone *et al.*, 2012; van der Wel *et al*, 2007).

Conserved *esx* loci contain five T7SS membrane components, *i.e.* EccB, EccC, EccD and EccE (Ecc standing for ESX conserved component) and 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 (Rosenberg *et al*, 2015). EccD is the most hydrophobic component with eleven TMDs and EccE is peripherally associated with the complex and 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 membrane complex of *M. tuberculosis* revealed the position of MycP within this complex, confirming the stabilizing role of MycP of 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, hereby covering approximately 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 PPE proteins to form a structurally similar heterodimer. Dimerization is mediated by

67 hydrophobic interactions resulting in a conserved four helix bundle structure. One of the secretion
68 partner proteins additionally contains a C-terminal YxxxD/E secretion signal that is important for
69 secretion.

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

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

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

92 ## Results

93

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

95 To be able to efficiently dissect the individual roles of T7SS components, we used the ESX-5 system of
96 the slow-growing, moderately thermophilic and pathogenic mycobacterial species *M. xenopi* (Fig. 1),
97 which was previously functionally reconstituted in *M. smegmatis* (Beckham *et al.*, 2017; van Winden *et*
98 *al.*, 2020). As *M. smegmatis* lacks a native ESX-5 system, the only ESX-5 substrates are those encoded
99 by the introduced *esx-5* locus, *i.e.* the Esx pair EsxM/EsxN and two PE/PPE pairs that are specific for *M.*
100 *xenopi* (Fig. 1). This is in contrast to ESX-5 systems in their natural hosts, which secrete multiple Esx
101 pairs and a large number of PE and PPE substrates (Ates, 2020; Gey van Pittius *et al.*, 2006). The limited
102 number of substrates secreted by the reconstituted system provides an excellent opportunity to
103 analyze their individual roles in T7SS functioning, bypassing any potential redundancy issues.

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

113 First, we analyzed the impact of the various gene deletions on the expression of the other
114 system components by SDS-PAGE and immunoblotting, using specific antisera for the different ESX-5
115 components. We used isolated cell envelope fractions of *M. smegmatis* expressing the different *esx-5*
116 plasmids to detect for membrane components (Fig. 2A) and whole cell lysates of the same strains to
117 assess for protein levels of the cytosolic components (Fig. 2B). Deletions of the genes coding for the
118 cytosolic components $EspG_5$ and $EccA_5$ did not affect the protein levels of any of the five inner
119 membrane components, *i.e.* $EccB_5$, $EccC_5$ (strep tagged), $EccD_5$, $EccE_5$ and $MycP_5$ (Fig. 2A). Furthermore,
120 in the absence of $EccA_5$, the protein levels of $EspG_5$ were not affected and *vice versa* (Fig. 2B).

121 Deleting the membrane components $EccB_5$ and $MycP_5$ did not affect protein levels of the four
122 other membrane components and the two cytosolic components. The absence of $EccE_5$ seems to affect
123 protein levels of $EccD_5$ and also of $EccA_5$, although the latter could be attributed to polar effects of the
124 gene deletion, as *eccA*$_5$ lies downstream of *eccE*$_5$ (Fig. 2A). Knocking out *eccD*$_5$ showed the most severe
125 effect on the protein levels of the other components. While $EccC_5$ was undetectable by immunoblotting

126 in the absence of $EccD_5$, the protein levels of the membrane components $EccB_5$ and $MycP_5$ were
127 reduced. Strikingly, also levels of the two cytosolic components were affected by the *eccD$_5$* deletion, as
128 $EspG_5$ could not be detected anymore and levels of $EccA_5$ were severely reduced. In conclusion, deletion
129 of the cytosolic components does not impact the protein levels of any of the other components. At the
130 same time, deleting *eccD$_5$* severely impacted the levels of all components.
131
132 **Only the five membrane components are required for full assembly of the ESX-5 membrane complex**
133 Next, we investigated which components play a role in the successful assembly of the ESX-5 membrane
134 complex. For this, we analyzed DDM-solubilized cell envelope fractions by blue native PAGE (BN-PAGE),
135 followed by immunoblotting using our anti-$EccB_5$ antibody or an antibody that recognized the Strep-
136 tag on $EccC_5$ (Fig 2C). Similar to previous observations (Beckham *et al.*, 2017), BN-PAGE analysis of
137 samples from mycobacteria expressing the complete *esx-5* locus resulted in three main complexes that
138 reacted with the anti-$EccB_5$ antibody, the largest complex representing the full complex of ~2 MDa and
139 two subcomplexes of ~900 kDa and ~700 kDa. Staining with the anti-Strep antibody showed a similar
140 immunoblot pattern. Taking into consideration the influence of the detergent, lipids and the Coomassie
141 dye on the size estimation of membrane proteins by BN-PAGE (Wittig *et al*, 2010), these three
142 complexes could represent the full hexameric complex, the dimeric subcomplex and an individual
143 protomer, respectively. In the absence of the cytosolic components $EccA_5$ and $EspG_5$, membrane
144 complex assembly was not affected, as the BN-PAGE immunoblot pattern in their absence was similar
145 as for the WT construct (Fig. 2C). Similarly, none of the substrate deletion plasmids showed any marked
146 differences in ESX-5 complexes, suggesting that substrates do not play a role in the assembly and/or
147 stability of the ESX-5 membrane complex. In line with this observation, a plasmid containing only the
148 five *esx-5* genes that code for inner membrane components resulted in membrane complex formation
149 similar to the intact *esx-5* locus (Fig. 2C).
150 Although the *ΔmycP$_5$* plasmid showed no major impact on the formation of the inner
151 membrane complex, an additional ~500 kDa $EccB_5$-containing complex was present, which was not
152 observed in the same sample when stained with an antibody against the Strep-tag on $EccC_5$ or in
153 samples with all five membrane components. These results were similar to previous observations in *M.*
154 *marinum*, where $MycP_1$ and $MycP_5$ of the ESX-1 and ESX-5 system, respectively, have been reported to
155 be involved in stabilizing its corresponding T7SS membrane complex (van Winden et al., 2016). Deletion
156 of any of the other four membrane components, *i.e.* $EccB_5$, $EccC_5$, $EccD_5$ and $EccE_5$, showed varying
157 effects on complex formation. In line with the observation that the *eccD$_5$* deletion severely affected
158 expression of the three other core components, no complex formation could be observed in this
159 condition using the anti-$EccB_5$ or anti-Strep antibody. This suggests that $EccD_5$ is crucial for complex

160 formation and/or stability, which is in line with its central location within the protomers of the
161 membrane complex. While deletion of either *eccB*$_5$, *eccC*$_5$ or *eccE*$_5$ did not drastically affect the
162 expression of the other membrane components (Fig. 2A), the assembly of the membrane complex, as
163 seen by BN-PAGE analysis, was affected (Fig. 2C). In the absence of EccB$_5$, only two faint subcomplexes
164 were observed. Similarly, the Δ*eccC*$_5$ construct showed again the ~500 kDa complex upon staining with
165 the EccB$_5$ antibody (Fig. 2C). Deleting *eccE*$_5$ showed a reduced amount of the full complex, using both
166 antibodies, which is in line with our previous observation that a lower number of full complexes could
167 be isolated in this condition (Beckham *et al.*, 2017). The additional ~500 kDa EccB$_5$-containing complex
168 was also present in the condition where EccE$_5$ was absent.
169 Taken together, this shows that optimal assembly of the ESX-5 membrane complex requires
170 only the five membrane components.
171
172 ESX-5 locus encoded PE/PPE proteins are required for secretion of Esx proteins
173 We previously observed that introduction of a plasmid that contains the complete *esx-5* locus of *M.*
174 *xenopi* in *M. smegmatis* resulted in the secretion of the plasmid-encoded ESX-5 substrate EsxN and also
175 of the *M. marinum* PPE18, which was introduced on an additional plasmid containing the *pe31/ppe18*
176 locus (Beckham *et al.*, 2017). This shows that the reconstituted system is functional. We therefore
177 tested next the impact of the various gene deletions on the ability of this system to secrete EsxN (Fig.
178 3AB). To check PPE protein secretion, we again introduced the plasmid containing the *pe31/ppe18* locus
179 of *M. marinum* (PPE18 containing a C-terminal HA-tag for detection).
180 While both EsxN and PPE18-HA were efficiently secretion with the WT construct, they were
181 also secreted in the absence of *eccA*$_5$, albeit at a reduced level. Deletion of the other cytosolic
182 component, *espG*$_5$, blocked the secretion of both PPE18-Ha and EsxN (Fig. 3A). Additionally, PPE18-HA
183 was not present in the bacterial pellet, suggesting that EspG$_5$ is essential for the stability of this protein,
184 as observed before (Ekiert & Cox, 2014; Korotkova *et al.*, 2014; Phan *et al.*, 2017) (Fig. 3A). As all five
185 membrane component deletions showed a disruption in membrane complex assembly, it is not a
186 surprise that they also abolished the secretion of both EsxN and PPE18-HA. In addition, deleting *eccD*$_5$
187 negatively affected the protein levels of both substrates (Fig 3A). In the absence of EsxM/EsxN,
188 secretion of PPE18-HA was abrogated, showing that the EsxM/N heterodimer is required for secretion
189 of the PE/PPE pair (Fig. 3A). Strikingly, in the absence of PE/PPE proteins encoded by the ESX-5 system,
190 which did not affect expression of any ESX-5 components nor assembly of the ESX-5 membrane
191 complex, both EsxN and PPE18 secretion was abolished (Fig. 3A). From this, we conclude that the
192 PE/PPE pairs of the *esx-5* locus are important for the secretion of the Esx substrates and at least one
193 other PPE protein.

194 To analyze the role of the individual PE/PPE pairs and of the putative methyltransferase that is
195 also encoded by the *esx-5* locus of *M. xenopi*, which was also deleted in the *Δpe/ppe* construct, we
196 created four complementation vectors containing either the entire deleted region (whole; Fig. 1), one
197 of the two *pe/ppe* gene pairs with the methyltransferase gene (*Δpe/ppe1* and *Δpe/ppe2*) or only the
198 two *pe/ppe* pairs without the methyltransferase gene (*Δmethyltransf.*). EsxN secretion could be rescued
199 by complementation with a plasmid containing the entire deleted region (Fig. 3B). The observed
200 secretion levels were reduced when compared to the levels in the WT constructs, as both introduced
201 plasmids contained the same origin of replication, thereby probably reducing plasmid copy numbers
202 and the overall protein levels of all *esx-5* encoded proteins, including EsxN. The same (reduced)
203 secretion levels were observed when we complemented with a plasmid that contains the entire deleted
204 region without the gene coding for the predicted methyltransferase, suggesting that this gene is not
205 required for secretion. However, deletion of either of the two *pe/ppe* pairs in the complementation
206 construct abrogated secretion of EsxN completely (Fig. 3B). This shows that both PE/PPE pairs are
207 required for EsxN secretion. Notably, complementing the *Δpe/ppe* plasmid with the PE31/PPE18-HA-
208 encoding plasmid did not restore secretion of EsxN (Fig. 3A). This shows that although PE31/PPE18-HA
209 is secreted in an ESX-5 dependent manner, it cannot take over the role of the *esx-5* locus-encoded
210 PE/PPE proteins in EsxN secretion. In conclusion, both the *esx-5* locus encoded PE/PPE proteins are
211 required for the secretion of EsxN and PPE18-HA by ESX-$5_{xen}$.
212

213 ## PPE but not Esx substrates localize to the cell envelope fraction

214 To track the substrates encoded by the *M. xenopi esx-5* locus, we tagged the first and the second PPE
215 with a Flag- and a Strep-tag, respectively, either at the N- or C-termini. To be able to detect EsxN more
216 efficiently, we added a C-terminal HA-tag to this substrate, as this has previously been shown not to
217 disrupt the secretion of its ESX-1 equivalent EsxA (Damen *et al*, 2020). Subcellular fractionation and
218 secretion analysis showed that a Flag-tag at the C-terminus of the first PPE protein partially affected
219 secretion of EsxN, whereas placing the tag at the N-terminus had no noticeable effect on EsxN secretion
220 (Fig. EV 1A). Fractionation of *M. smegmatis* cells showed that the first PPE with the N-terminal Flag-tag
221 localized to the cell envelope fraction and also to the cytosolic fraction (Fig. EV 1A). Similarly, this
222 substrate was secreted and found both in the culture filtrate and in the Genapol X-080 extractable
223 surface fraction. This correlated well with the surface-localization of the Flag-tagged PPE proteins, as
224 seen by flow cytometry analysis. Here, the N-terminal variant showed a much higher signal on the
225 surface than the C-terminal positioned tag (Fig. EV 1B).

226 The second PPE could not be detected, when tagged with a Strep-tag at the N-terminus, while
227 placing the same tag to the C-terminus showed sufficient protein levels (Fig. EV 1A). With both

228 constructs, EsxN was efficiently secreted to the culture supernatant (Fig. EV 1A). While untagged EsxN
229 could be detected by the anti-EsxN antibody, we could detect EsxN with a C-terminal HA-tag only using
230 the anti-HA antibody (Fig. EV 1A). Similar to the first PPE, secretion of the second PPE was observed
231 into the culture supernatant as well as on the bacterial surface. Following fractionation of cells
232 expressing the second PPE with a C-terminal Strep-tag, only a small portion of the tagged substrate was
233 in the cytosolic fraction, while the major portion of the protein localized to the cell envelope fraction
234 (Fig. EV 1A).

235 With this information in hand, we made a final construct in which we removed the previously
236 introduced Strep-tag at the C-terminus of $EccC_5$ and modified the substrate genes, so that they encoded
237 for a Flag-tag at the N-terminus of the first PPE, a Strep-tag at the C-terminus of the second PPE and an
238 HA-tag at the C-terminus of EsxN. We analyzed protein secretion by *M. smegmatis* carrying this
239 construct and grown in the presence or absence of Tween-80, a mild detergent used as anti-clumping
240 agent for growing mycobacteria in liquid culture (Fig. EV2). While EsxN was present in the culture filtrate
241 in both conditions, both PPE proteins were found in the culture filtrate and not in the Genapol X-080
242 extractable surface fraction, when cells were grown in the presence of Tween-80, while in the absence
243 of Tween-80, these proteins were exclusively found in the surface fraction. This suggests that the
244 presence of the detergent triggers the release of these proteins from the cell surface into the culture
245 medium. To test if after Genapol-extraction a portion of the two PPE proteins are still cell envelope
246 associated, we labelled cells, carrying the construct expressing all tagged substrates, after treatment
247 with Genapol X-080, with anti-Flag, -Step and -HA antibodies. EsxN could not be detected via its C-
248 terminal HA-tag by subsequent flow cytometry, which is in line with the observation that EsxN is mainly
249 secreted into the culture medium. Interestingly, using anti-Flag and anti-Strep antibodies similar levels
250 of surface labeling could be observed as compared to cells that were not extracted with Genapol X-080
251 (Fig. 4BC). This suggests that the major portion of the two PPE proteins that are surface accessible
252 remain cell envelope associated upon detergent treatment.

253 To characterize their cell envelope association further, we isolated cell envelope fractions from
254 cells with the final construct, grown in the absence of Tween-80 and removed their surface extractable
255 proteins using Genapol X-080. We then subjected the isolated cell envelope fractions to solubilization
256 with a range of detergents, some of which have been used previously for characterization of
257 mycobacterial membrane proteins (Hoffmann *et al*, 2008; Målen *et al*, 2010). While our inner
258 membrane protein controls, $EccC_5$ and FstH could be extracted by the nonionic detergents DDM, beta-
259 octylglucoside and Triton X-100, and the anionic detergent SDS, but not by the milder nonionic
260 detergent Genapol X-080 or zwitter ionic detergent CHAPSO, the first PPE substrate could be

261 solubilized, partially or fully, by all these detergents. In contrast, the second PPE protein could not be
262 solubilized by any of these detergents, with the exception of SDS (Fig. 4A).

263 Together, we conclude that a major portion of both *esx-5*-locus encoded PPE proteins localize
264 to the mycobacterial cell envelope, where they are surface exposed. In addition, the observation that
265 a major subpopulation of the second PPE protein cannot be extracted from the cell envelope with
266 detergents that are able to solubilize inner membrane proteins indicates that this protein is an integral
267 membrane protein. Therefore, this protein could potentially form a channel that mediates protein
268 transport across the mycomembrane.

269

## Discussion

In this study, we used a functional ESX-5 system reconstituted in *M. smegmatis* to define a minimal functional unit for ESX-5, by analyzing the role of each component and substrate pair in protein levels, membrane complex formation and secretion. The obtained results in this study are summarized in Table 1.

Deletion of membrane components affected assembly of the complex to various extents, as judged by BN-PAGE analysis. Deleting *mycP$_5$* or *eccE$_5$* showed a relatively mild effect on the overall stability of the complex. Deletion of *eccB$_5$* affected membrane complex formation, especially for the higher molecular weight species, which is in line with previous findings that EccB$_5$ is involved in complex multimerization (Bunduc *et al.*, 2021; Famelis *et al.*, 2019). In the absence of EccC$_5$, membrane complex formation was almost completely abolished, with this construct exhibiting only a ~500 kDa subcomplex containing EccB$_5$, observed also in the absence of MycP$_5$ or EccE$_5$ but not seen with the WT construct. The most striking phenotype was observed in the absence of EccD$_5$, which had a large negative effect not only on the formation of the membrane complex, but also on the expression of all other T7SS components, including cytosolic components and substrates. The fact that the *esx-5* gene cluster is introduced in *M. smegmatis* that lacks ESX-5 itself makes it unlikely that these reduced protein levels are caused on a transcriptional level by a negative feedback loop, as has been described for the ESX-1 system (Abdallah *et al*, 2019; Bosserman *et al*, 2017; Chen *et al*, 2016). This indicates that the lack of EccD$_5$ triggers a domino-effect on the stability of the other proteins. In its absence, the membrane complex cannot be assembled, resulting in the degradation of all other membrane components. Subsequently, in the absence of any membrane (sub)complexes, cytosolic components also become unstable, hereby destabilizing the substrates.

Individual substrate mutants or deletions in either of the two cytosolic chaperones showed no defect in membrane complex formation, as analyzed by BN-PAGE. Complex assembly required only the five conserved membrane components, as a construct encoding only these components showed successful complex assembly. Although complex formation did not appear to be affected by BN-PAGE analysis, conformational changes could occur at the membrane complex level upon chaperone or substrate-binding.

By systematic deletions we have defined the minimal functional T7SS ESX-5 unit, which requires the five membrane components, EspG$_5$, both PE/PPE pairs that are encoded by the *esx-5* locus and an Esx heterodimer. Naturally, functionality was tightly connected with the successful assembly of the membrane complex, as none of the deletions in membrane components showed secretion of EsxN or PPE18-HA. EspG$_5$ proved to be critical for successful secretion of both PE/PPE and EsxM/N proteins,

304 similar to previous findings (Korotkova *et al.*, 2014). The system without $EccA_5$ was still functional,
305 although to a lower extent. EccA proteins have been shown to be required for secretion in some but
306 not all studies (Bottai *et al*, 2012; Joshi *et al*, 2012; Phan *et al.*, 2018).

307 Deletion of the SAM-methyltransferase gene located between the two *pe/ppe* gene pairs of the
308 *esx-5* cluster was not essential for a functional system. Methyltransferases are not often associated
309 with *esx-5* or other T7SSs loci, suggesting that the presence of this gene could be a coincidence. In
310 contrast, we showed that secretion of the Esx heterodimers by ESX-5 is dependent on the locus
311 encoded PE/PPE proteins. Additionally, the reverse was also true, *i.e.* PE/PPE proteins require Esx
312 heterodimers to be secreted. This is in line with a recent observation for ESX-1 in *Mycobacterium*
313 *marinum*, where secretion of EsxA is dependent on the co-secretion of PPE68 that is encoded by the
314 same gene cluster (Damen *et al*, 2022). Notably, PPE68 was observed to be degraded on the cell surface
315 upon export. Since PE31/PPE18 was not able to complement secretion of the Esx pair, our results are
316 in line with a model in which Esx and PE/PPE proteins are specifically paired for their co-secretion. The
317 observation that both *esx-5* encoded PE/PPE proteins and EsxM/EsxN are required for PPE18-HA
318 secretion shows that both substrate classes are necessary for ESX-5 functionality.

319 The interdependency of PE/PPE and Esx substrates could be explained by a previously proposed
320 dual recognition event by the EccC ATPase (Bunduc *et al*, 2020b). EccC interacts with Esx proteins via
321 its third NBD and potentially drives the multimerization of its soluble domains (Rosenberg *et al.*, 2015).
322 This step is needed for completion of the active site of the first crucial NBD by neighboring arginine
323 finger motifs, called R-fingers (Bunduc *et al.*, 2020b). A second recognition step, which activates the
324 NBD1, takes place at the NBD1/linker 2 interface. The linker 2 domain that connects NBD1 and NBD2,
325 might be a recognition site for PE/PPE proteins, as this region has been shown to be involved in the
326 species-specific secretion of the ESX-5 dependent PE_PGRS proteins in *M. marinum* (Bunduc *et al.*,
327 2020b).

328 Interdependency could also take place beyond the T7SS membrane complex. The dimensions
329 of the core membrane complex dictates that this complex spans only the inner membrane. How protein
330 transfer takes place beyond the inner membrane is unknown as there is no clear data on the identity
331 of the mycomembrane channel or components. As our reconstituted ESX-5 system is functional in *M.*
332 *smegmatis*, the proteins that drive secretion over this second membrane are likely encoded by the
333 introduced *esx-5* cluster. Within the $esx\text{-}5_{xen}$ locus, the proteins that have thus far been regarded as
334 substrates are the only proteins that could mediate translocation through the mycomembrane, as all
335 other components are localized either in the inner membrane or in the cytosol. In addition, in recent
336 years it has become clear that various PE/PPE proteins are involved in the uptake of nutrients and
337 localize to the cell envelope, with PPE51 as the main example (Ates *et al.*, 2015; Korycka-Machala *et al*,

338 2020; Wang *et al*, 2020). Indeed, in our reconstituted system both PPE substrates also localized to cell
339 envelope to various degrees. The first PPE protein was well secreted and localized to the cell envelope
340 and cytosol in relatively equal amounts, whereas the second PPE substrate localized mainly to the cell
341 envelope compartment, with only a minimal portion remaining in the cytosol. The second PPE protein
342 also showed to be largely non-extractable by the detergents that solubilize inner membrane proteins.
343 Notably, this is also an observed feature of known or predicted integral mycomembrane proteins (Heinz
344 & Niederweis, 2000; Speer *et al*, 2015), suggesting this PPE protein might be integrated in the
345 mycomembrane. We therefore propose two working models. In the first model, the roles of the two
346 *esx-5* locus encoded PPE proteins in secretion are different, considering the differences in localization
347 and membrane association. Here, the first PPE protein could play a role in activation of the inner
348 membrane complex and co-secretion together with the EsxM/N heterodimer, similar as for the ESX-1
349 dependent Esx and PE/PPE substrates (Damen *et al.*, 2022) , while the second PPE protein could form
350 the channel for protein translocation over the mycomembrane. In the second model, the two PPE
351 proteins form the mycomembrane channel together, with the second PPE protein creating the integral
352 part and the first PPE being the peripheral portion of the complex. As these PPE proteins are specific
353 for *M. xenopi*, further studies are required to determine whether the *esx-5*-associated PPE proteins of
354 other mycobacterial species, such as *M. tuberculosis*, have similar properties.

355 ## Materials and methods

356

357 ### Bacterial strains and growth conditions

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

362

363 ### Molecular cloning

364 Cloning was performed using *E. coli* Dh5α and restriction enzymes from New English Biolabs.
365 Polymerase chain reaction (PCR) products were amplified with IProof DNA polymerase from BioRad and
366 fused in multi-product reactions with InFusion HD from TakaraBio. For construction of plasmid
367 derivatives, genes were deleted, either completely if they were in a single operon or ~100-150 bp were
368 left at the C-terminus, if these were in an operon with other downstream genes. Generally, genes were
369 deleted using the WT ESX-$5_{xen}$ plasmid as template using the closest unique restriction sites up- and
370 downstream of the gene of interest and amplification of two or more PCR fragments with compatible
371 18bp ends, where the gene of interest was omitted. Because for different deletions the same restriction
372 sites were used, some primers were used for construction of multiple derivatives. For a complete
373 overview of restriction enzymes, primers, plasmids and cloning strategies see Table S1, Table S2 and
374 Table S3.

375

376 ### Protein secretion and immunoblot analysis

377 *M. smegmatis* $MC^2155$ with plasmids coding for ESX-$5_{xen}$ WT or its derivatives were grown in LB liquid
378 medium with 0.05% Tween 80 and appropriate antibiotics at 37°C and 90 RPM until mid-log phase.
379 Cells were sub-cultured at an $OD_{600}$ of 0.05 in LB liquid media under the same conditions for 12-16
380 hours until an $OD_{600}$ of 0.8-1 was reached. Cultures were spun down, supernatants were passed through
381 0.2 µm filters and precipitated with trichloroacetic acid (TCA), resulting in culture supernatant fractions.
382 Cell pellets were broken via bead-beating with glass beads, resulting in whole cell lysates or bacterial
383 pellet fractions. SDS loading buffer was added to all samples, boiled and loaded on SDS-PAGE gels (10%
384 or 16% depending on the protein size), transferred to nitrocellulose membranes and stained with
385 appropriate antibodies. Antibodies against $EccA_5$, $EccB_5$, $EccD_5$, $EccE_5$, $EspG_5$, FtsH, $MycP_5$, EsxN
386 (Mtb9.9) and GroEL2 have been described previously (Houben *et al.*, 2012b); HA- and Strep-antibodies
387 were purchased from Thermo Scientific (2-2.2.14) and Novusbio (NBP2-41073).

388

389 Cell envelope isolation

390 *M smegmatis* $MC^2155$ with the various ESX-$5_{xen}$ plasmids were grown as above to an $OD_{600}$ of ~1.5. Cells
391 were washed in PBS, resuspended in buffer (50 mM Tris-HCl, 300 mM NaCl and 10% glycerol) and
392 broken by passing two times through a high-pressure homogenizer (Stansted). Unbroken cells were
393 pelleted at 5000xg. Cell envelopes were separated from the soluble fraction by ultracentrifugation at
394 150.000xg. After ultracentrifugation, pellets were washed and resuspended in buffer and snap frozen
395 in liquid nitrogen. Where stated, after culture harvesting, cells were incubated with mixing at room
396 temperature with 0.5% Genapol X-80 for 30 minutes, to remove the capsular layer on the surface of
397 bacteria. Subsequently, cells were centrifuged at 5000xg, washed one time with buffer and fractionated
398 as mentioned above.

399

400 BN-PAGE

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

406

407 Flow cytometry analysis.

408 *M. smegmatis* expressing different plasmids was grown to an OD600 of 0.8 to 1.2 in LB with or without
409 Tween 80. Bacteria were pelleted, washed with PBS with 1% bovine serum albumin (BSA; Sigma), and
410 incubated for 2h with antibodies recognizing the Flag-tag (F-1804; Sigma-Aldrich), Strep tag (NBP2-
411 41073; Novusbio) or influenza virus hemagglutinin tag (2-2.2.14; Thermo Scientific). After washing with
412 PBS with 1% BSA, bacteria were incubated with secondary goat anti-mouse IgG (A-21235;
413 ThermoFischer) or secondary goat anti-rabbit IgG (A-21244; ThermoFischer) conjugated to Alexa 647
414 antibodies for 30min. After washing with PBS with 1% BSA, bacteria were analyzed by flow cytometry
415 (Attune NxT; ThermoFisher). As a control, bacteria were incubated only with the secondary antibodies.

416

417 Acknowledgements

418 Conflict of interest

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535 2149-2161

536

537

538 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*$_5$ | ++ | ++ | + | + | + | - | - |
| Membr. comp. | ++ | n.a. | n.a. | + | ++ | n.a. | - |
| Δ*esxM/N* | ++ | ++ | n.a. | + | ++ | n.a. | - |
| Δ*pe/ppe* | ++ | ++ | + | + | ++ | - | - |
| Δ*substrates* | ++ | ++ | n.a. | + | ++ | n.a. | - |

539 n.a., not analyzed.

540 **Figure legends**

541

542 **Figure 1: Genetic organization of the *esx-5* locus of *M. xenopi* RIVM700367 and several of its**
543 **derivatives**

544 Depicted are the complete gene cluster, the cluster derivatives, in which more than one gene was
545 deleted and the gene organization for the *pe*/*ppe* region complementation plasmid.

546

547

548 **Figure 2: Protein expression and membrane complex formation for ESX-5 mutants**

549 (A) SDS-PAGE and immunoblot analysis of cell envelope fractions of *M. smegmatis* carrying the ESX-
550 $5_{xen}$ plasmid or its derivatives using antibodies reactive against the four membrane components $EccB_5$,
551 $EccD_5$, $EccE_5$ and $MycP_5$ or antibodies directed against a Strep-tag linked to the membrane component
552 $EccC_5$. Antibodies against the unrelated membrane protein FtsH were used as loading control. Notably,
553 $MycP_5$ is processed in one of the loops of its protease domain (van Winden *et al.*, 2016), resulting in
554 two immuno-reactive bands (arrow heads). The high hydrophobicity of $EccD_5$ compromises its
555 biochemical analysis, resulting in its variable detection by SDS-PAGE and immunoblotting. (B) SDS-
556 PAGE and immunoblot analysis of whole cell lysates of *M. smegmatis* carrying the ESX-$5_{xen}$ plasmid or
557 its derivatives using antibodies reactive against the cytosolic ESX-5 components $EccA_5$ and $EspG_5$.
558 Antibodies against GroEL2 were used as loading control. (C) BN-PAGE and immunoblot analysis of
559 DDM-solubilized cell envelope fractions of *M. smegmatis* carrying the ESX-$5_{xen}$ plasmid or its
560 derivatives using antibodies against $EccB_5$ and the Strep-tag introduced on $EccC_5$. * denotes the ~500
561 KDa subcomplex seen in the *eccC*$_5$, *eccE*$_5$ and *eccD*$_5$ mutants.

562

563 **Figure 3: Secretion analysis of *M. smegmatis* carrying the ESX-$5_{xen}$ plasmid or its derivatives**

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

571

572 **Figure 4: Solubilization of membrane bound PPE proteins**

573 (A) SDS-PAGE and immunoblot or Coomassie blue stain analysis of cell envelope fractions isolated
574 from cells treated with Genapol X-80 and solubilized with the mentioned surfactants. Concentrations
575 for the detergents are: 0,25% DDM, 1% n-Octyl-β-D-Glucopyranoside, 2% Triton X-100, 2% SDS, 0.5%
576 Genapol X-80 and 5% CHAPSO. Fractions were loaded in equal amounts. Antibodies against $EccB_5$ and
577 FtsH were used as inner membrane controls. S, soluble fraction; NS, non-soluble fraction. (B) and (C)
578 Surface exposure of substrates on whole cells was measured by flow cytometry of cells grown in the
579 presence of Tween-80 without (B) or with (C) surface protein extraction with Genapol X80 after culture
580 harvesting.
581

582 **Expanded View Figure 1: Subcellular fractionation of *M. smegmatis* carrying the *esx-5$_{xen}$* plasmid**
583 **expressing tagged ESX-5 substrates**

584 (A) Cells expressing the *M. xenopi esx-5* locus encoding substrates with tags at the N or C- termini were
585 grown in the presence of 0.05% Tween 80, after which supernatants (S) were separated from bacterial
586 cells (P). Half these cells were treated with Genapol X-80 to extract surface proteins, producing the
587 Genapol supernatant (GS) and Genapol pellet (GP). Both Genapol-treated and nontreated cells were
588 subsequently fractionated into total (T), cytosol (Cyt) and cell envelope (CE) fractions. Fractions were
589 loaded in a ratio of 1:1:1:2:2 for T:Cyt:CE:GS:S. Antibodies against FtsH (inner membrane protein) was
590 used as fractionation controls. Background bands corresponding to the protein ladder are marked
591 with *. (B) Surface exposure of substrates on whole cells was measured by flow cytometry of cells
592 grown in the presence of Tween-80.

593

594 **Expanded View Figure 2: Secretion analysis of *M. smegmatis* carrying the ESX-5$_{xen}$ plasmid**
595 **expressing tagged substrates**
596 SDS-PAGE and immunoblot analysis of secreted fractions (culture supernatants) and whole cell lysates
597 (bacterial pellets) of *M. smegmatis* carrying the ESX-$5_{xen}$ plasmid encoding the first PPE substrate with
598 a Flag-tag at the N-terminus, the second PPE protein with a Strep-tag at the C-terminus and EsxN with
599 a HA-tag at the C-terminus. Antibodies against GroEL2 were used as lysis control. Cells were grown in
600 the presence or absence of Tween-80, after they were harvested half was treated with Genapol-X-80
601 to extract surface proteins. P, bacterial pellet; S, culture supernatant; GS, Genapol supernatant; GP,
602 Genapol pellet.
603

604 **Supplementary table 1: List of plasmids used in this study**

605

606 **Supplementary table 2: Primers used in this study for molecular cloning**

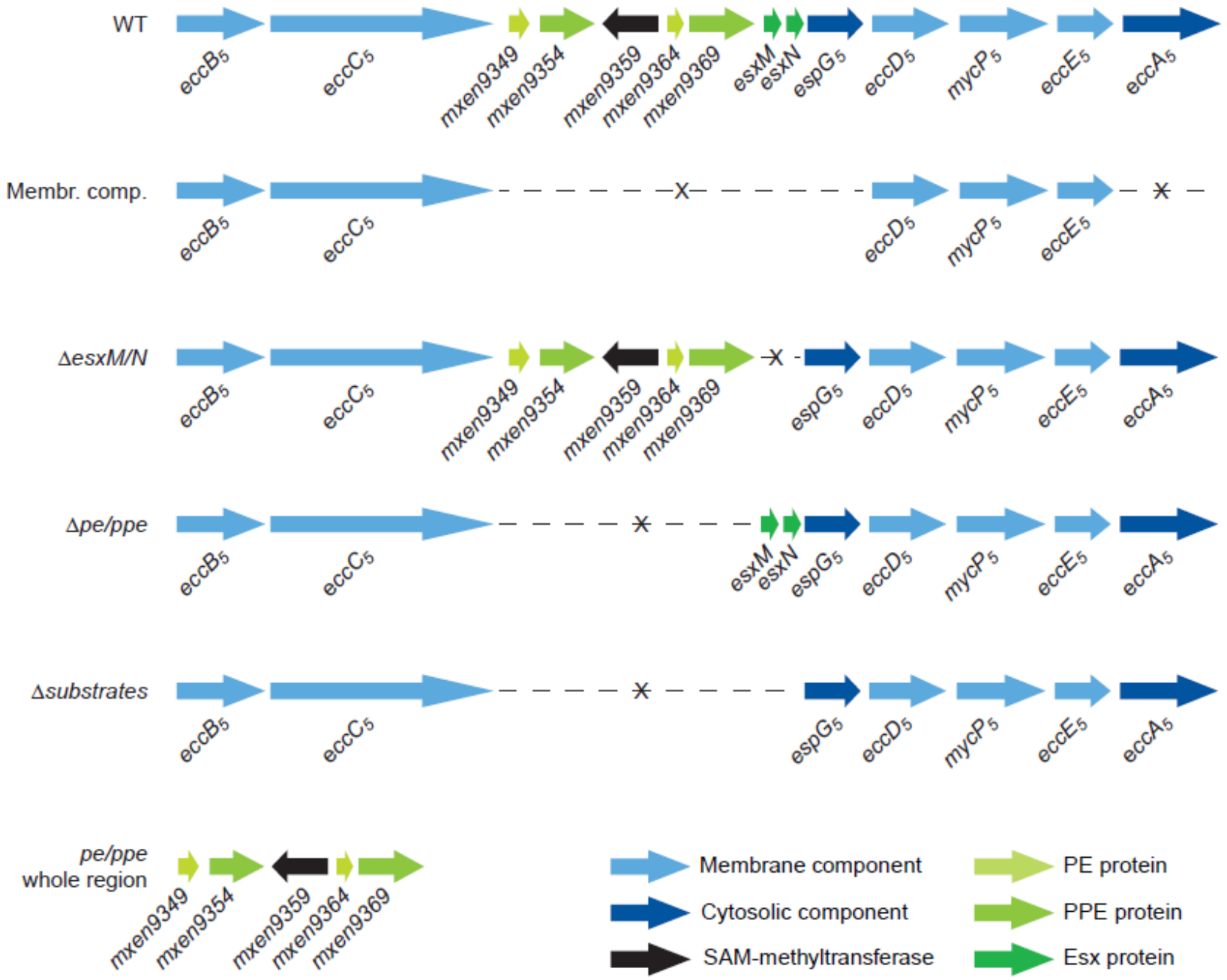
607

608 **Supplementary table 3: Overview of molecular cloning strategies for generating the plasmids shown**

609 **in Table S1 and using primers shown in Table S2.**

610

Figure 1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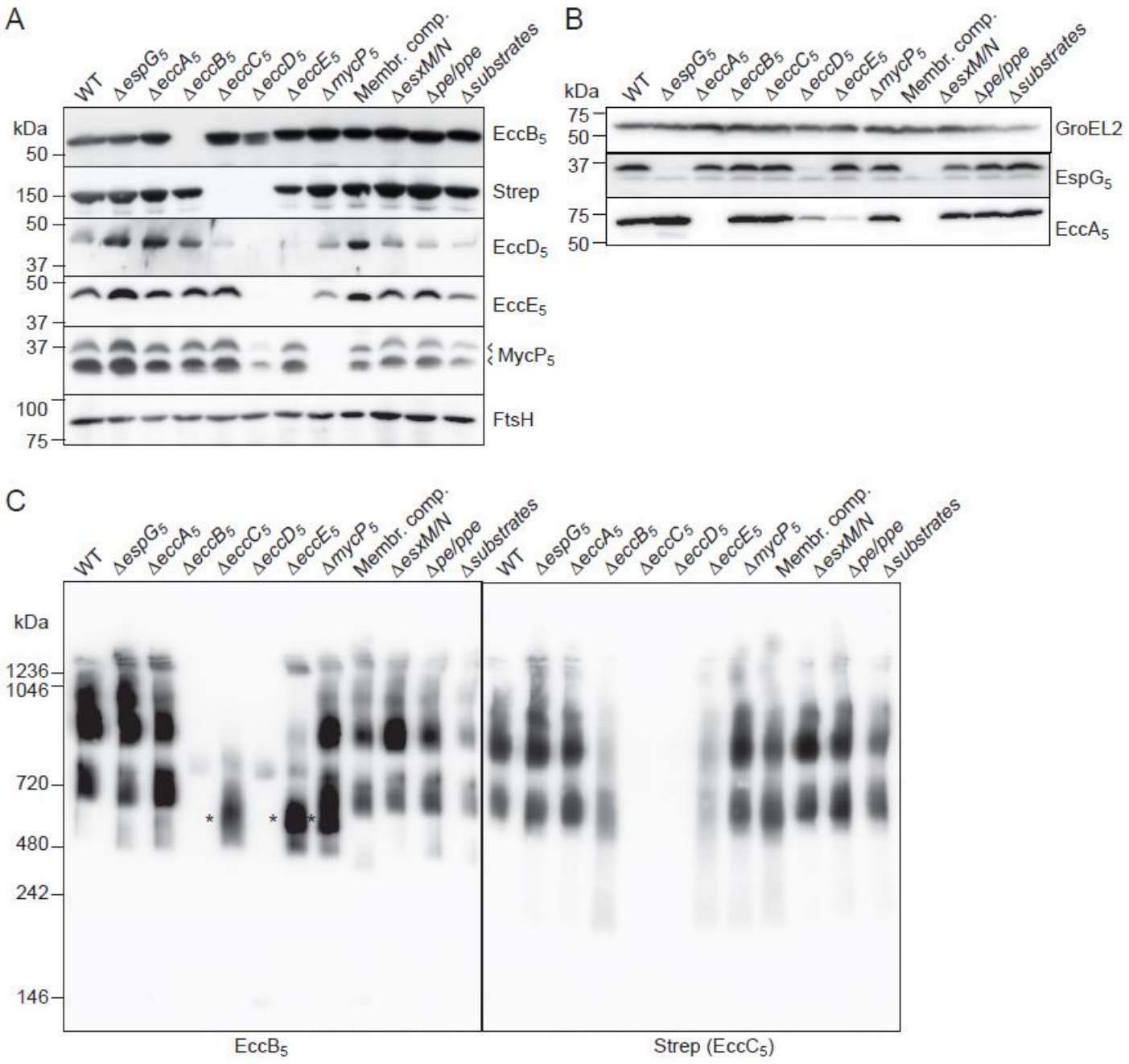
eccC5
mxen9349
mxen9354
mxen9359
mxen9364
mxen9369
esxM
esxN
espG5
eccD5
mycP5
eccE5
eccA5
Membr. comp.
ΔesxM/N
Δpe/ppe
Δsubstrates
pe/ppe
whole region
Membrane component
Cytosolic component
SAM-methyltransferase
PE protein
PPE protein
Esx protein

Figure 2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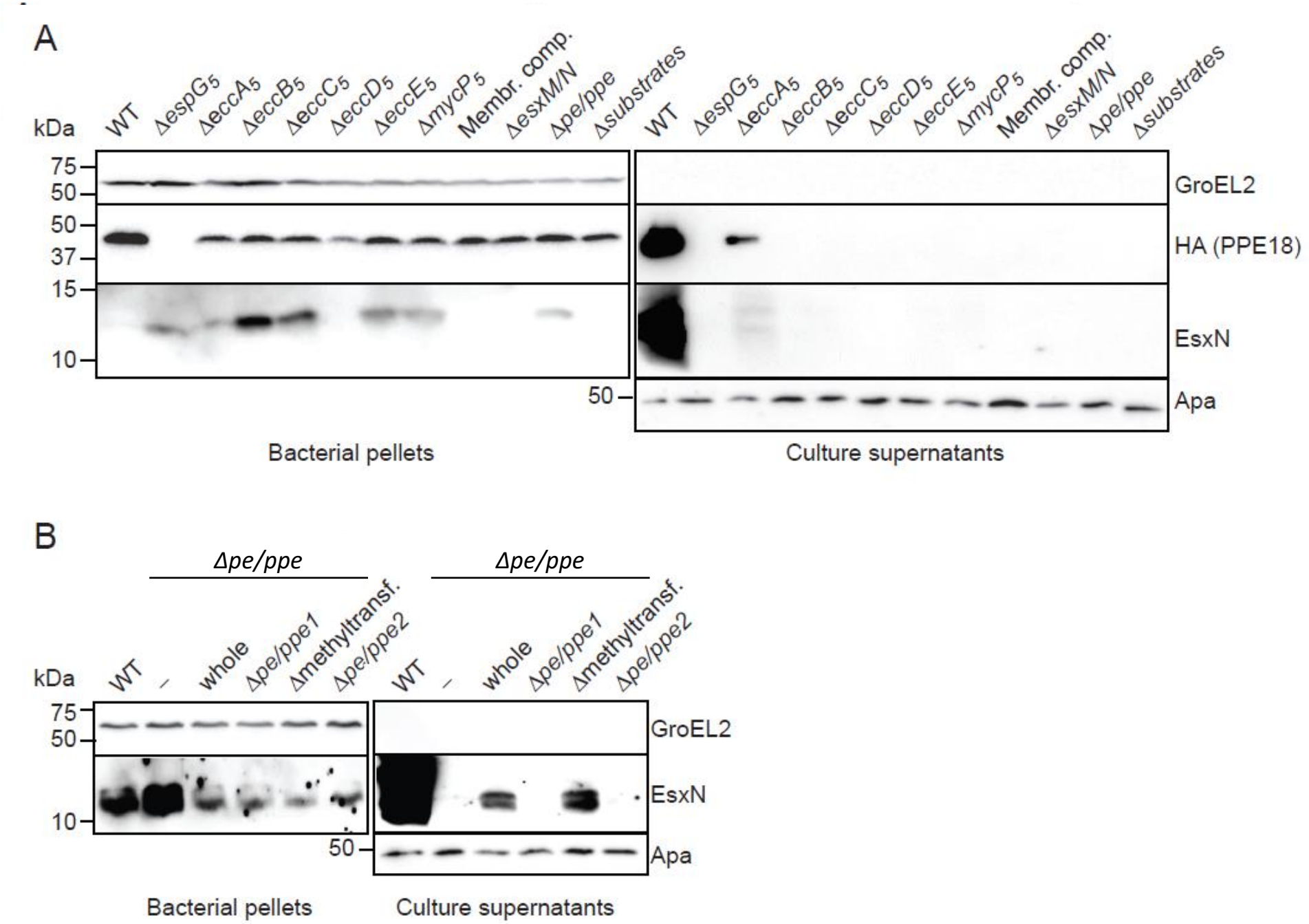
WT
ΔespG5
ΔeccA5
ΔeccB5
ΔeccC5
ΔeccD5
ΔeccE5
ΔmycP5
Membr. comp.
ΔesxM/N
Δpe/ppe
Δsubstrates
kDa
50
150
50
37
50
37
37
100
75
EccB5
Strep
EccD5
EccE5
MycP5
FtsH
B
kDa
75
50
37
75
50
GroEL2
EspG5
EccA5
C
kDa
1236
1046
720
480
242
146
EccB5
Strep (EccC5)

Figure 3


A
kDa
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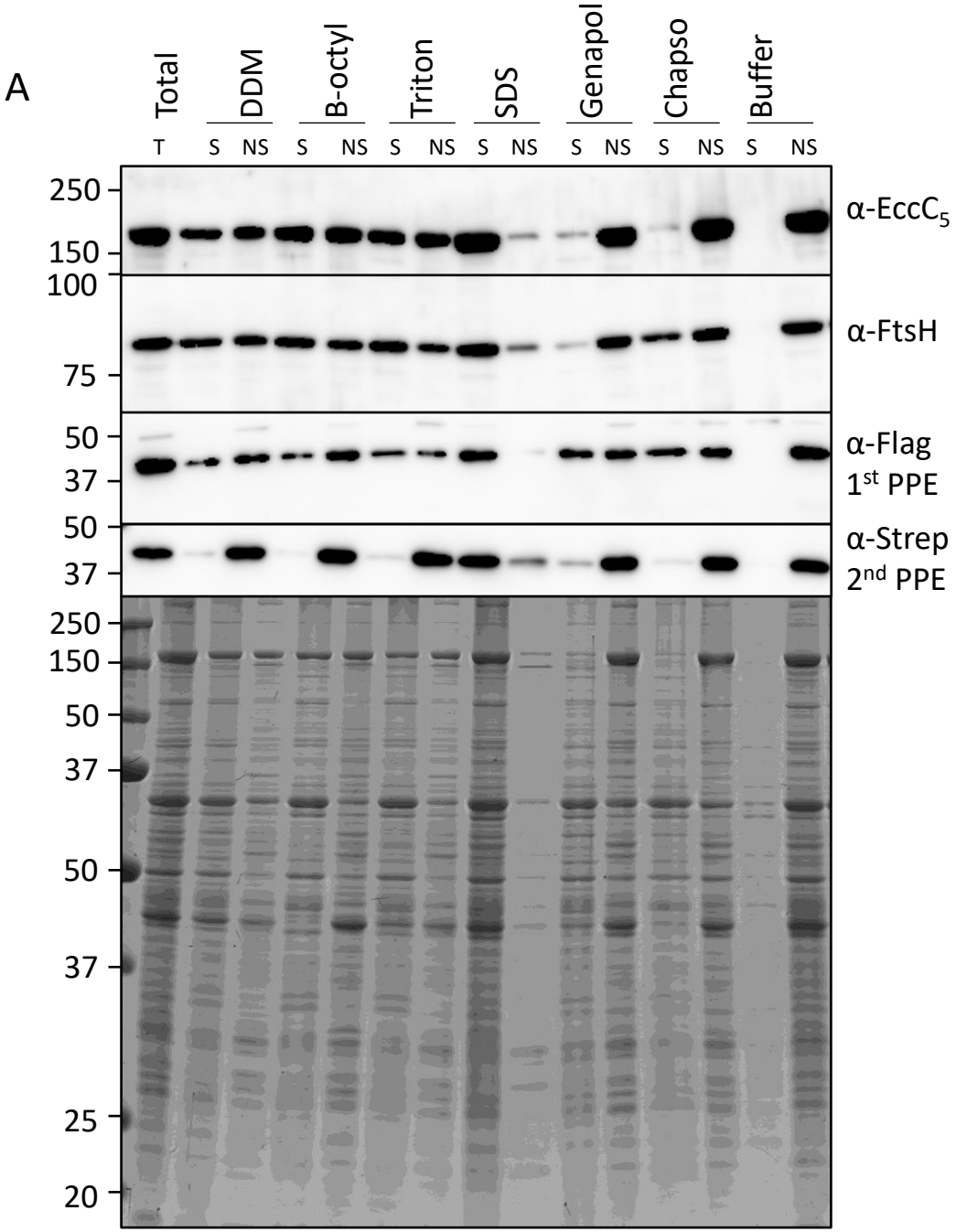
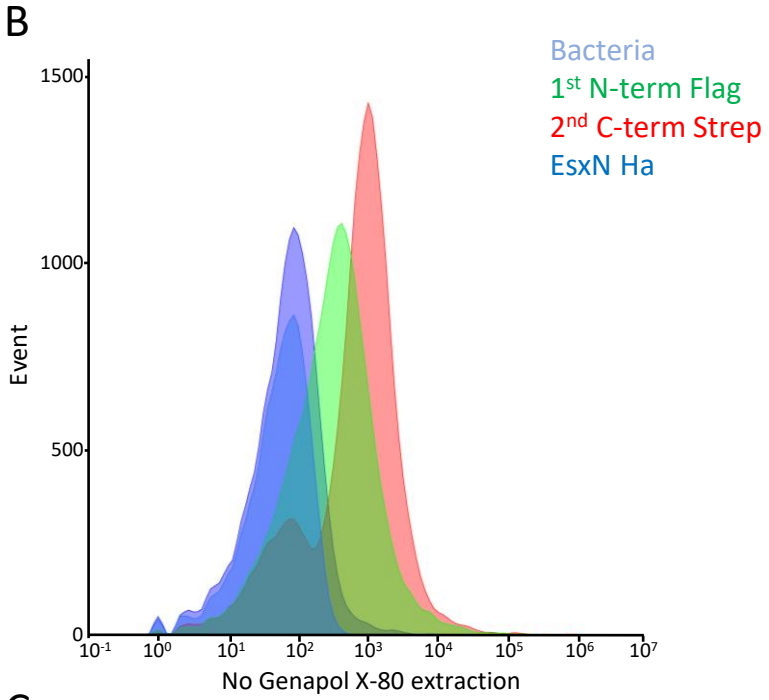
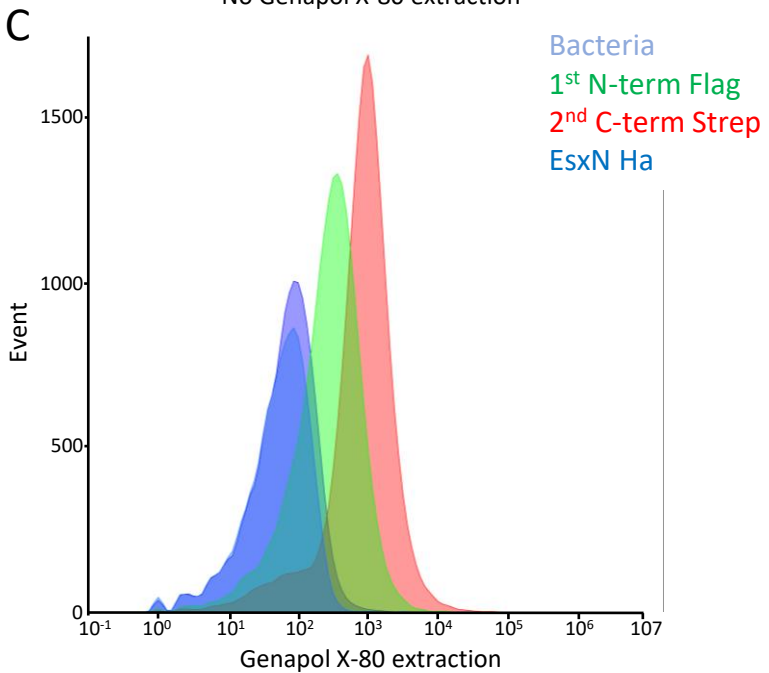
ΔeccA5
ΔeccB5
ΔeccC5
ΔeccD5
ΔeccE5
ΔmycP5
Membr. comp.
ΔesxM/N
Δpe/ppe
Δsubstrates
75
50
50
37
15
10
50
GroEL2
HA (PPE18)
EsxN
Apa
Bacterial pellets
Culture supernatants
B
Δpe/ppe
Δpe/ppe
kDa
WT
–
whole
Δpe/ppe1
Δmethyltransf.
Δpe/ppe2
75
50
10
50
GroEL2
EsxN
Apa
Bacterial pellets
Culture supernatants

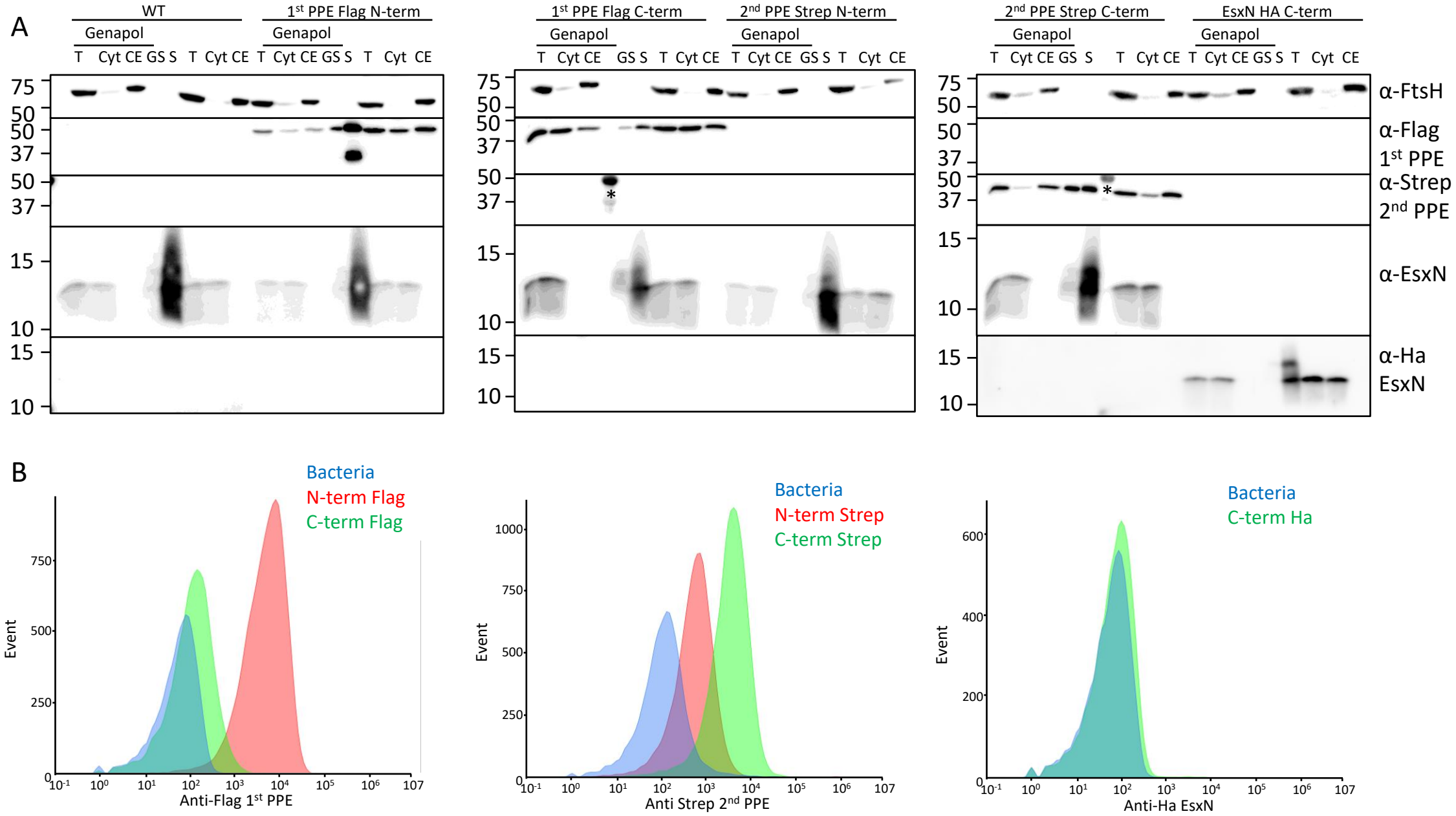
Figure 4


A
Total
DDM
B-octyl
Triton
SDS
Genapol
Chapso
Buffer
T
S
NS
250
150
100
75
50
37
25
20
α-EccC5
α-FtsH
α-Flag
1st PPE
α-Strep
2nd PPE


B
Bacteria
1st N-term Flag
2nd C-term Strep
EsxN Ha
Event
No Genapol X-80 extraction


C
Bacteria
1st N-term Flag
2nd C-term Strep
EsxN Ha
Event
Genapol X-80 extraction

# Expanded View Figure 1



Expanded View Figure 2

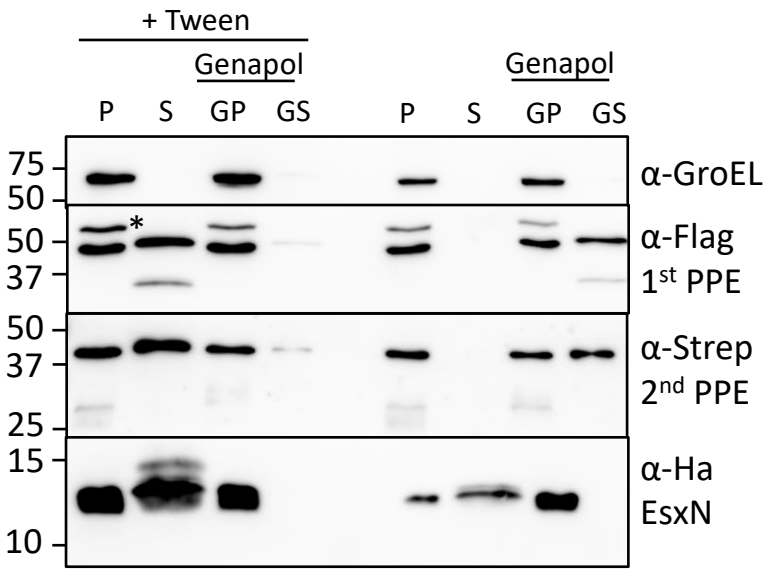


**Table S1**: List of plasmids used in this study

| Nr. | Construct | Source |
|---|---|---|
| 1. | pMV ESX-5xen | Beckham et al., 2017 |
| 2. | pMV ESX-5xen Δ$eccB_5$ | this study |
| 3. | pMV ESX-5xen Δ$eccC_5$ | this study |
| 4. | pMV ESX-5xen Δpe/ppe | this study |
| 5. | pMV ESX-5xen ΔesxM/N | this study |
| 6. | pMV ESX-5xen Δsubstrates | this study |
| 7. | pMV ESX-5xen Δ$espG_5$ | this study |
| 8. | pMV ESX-5xen Δ$eccD_5$ | this study |
| 9. | pMV ESX-5xen Δ$mycP_5$ | van Winden et al., 2020 |
| 10. | pMV ESX-5xen Δ$eccE_5$ | this study |
| 11. | pMV ESX-5xen Δ$eccA_5$ | this study |
| 12. | pMV ESX-5xen membrane components | this study |
| 13. | pSMT3 PE/PPE whole region | this study |
| 14. | pSMT3 PE/PPE Δ1$^{st}$_pe/ppe | this study |
| 15. | pSMT3 PE/PPE Δmethyltransferase | this study |
| 16. | pSMT3 PE/PPE Δ2$^{nd}$_pe/ppe | this study |
| 17. | pMV ESX-5xen 1$^{st}$ PPE Flag N-term | this study |
| 18. | pMV ESX-5xen 1$^{st}$ PPE Flag C-term | this study |
| 19. | pMV ESX-5xen 2$^{nd}$ PPE Strep N-term | this study |
| 20. | pMV ESX-5xen 2$^{nd}$ PPE Strep C-term | this study |
| 21. | pMV ESX-5xen EsxN-HA | this study |
| 22. | pMV ESX-5xen 1$^{st}$ PPE Flag N-term, 2$^{nd}$ PPE Strep C-term, EsxN-HA, No EccC Strep. | this study |

**Table S2**: 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 1$^{st}$ PPE flag C 1 RV | TGTCGTCATCGTCTTTGTAGTCGCCGGCGAACGGTGGGCGTG |
| 24. | Xen 1$^{st}$ PPE Flag C 2 FW | ACAAAGACGATGACGACAAGTAGCAACACGCGAAGCGCCG |
| 25. | Xen tag substr 2 RV | TGCATCTCGACCGAATACGTAAGTGAAA |
| 26. | Xen 2$^{nd}$ PPE Strep C 1 RV | TTCAAATTGGGGATGCGACCATGATCCTCCACCGGCGTAGACCGGGCG |
| 27. | Xen 2$^{nd}$ PPE Strep C 2 FW | TCGCATCCCCAATTTGAAAAATAGGAAAGGGGGCCAGCCG |
| 28. | Xen EsxN-HA 1 RV | TAATCAGGAACATCATACGGATAGGCCCAGCTGGAGCCGAC |
| 29. | Xen EsxN-HA 2 FW | CGTATGATGTTCCTGATTATGCTTAACAACCAACCGCAGGCGC |
| 30. | Xen 1$^{st}$ PPE flag Nterm 1 RV | CGAACCTCCCGAACCTCCCTTGTCGTCATCGTCTTTGTAGTCCATTAGTACTCTCCTTAGCCCGCCG |
| 31. | Xen 1$^{st}$ PPE Flag Nterm 2 FW | GGAGGTTCGGGAGGTTCGGTGCTGGACTTCGGGGCATTTC |
| 32. | Xen 2$^{nd}$ PPE Strep Nterm 1 RV | CGAACCTCCCGAACCTCCTTTTTCAAATTGGGGATGCGACCACATCTAGAGAAGCCCCCGCC |
| 33. | Xen 2$^{nd}$ PPE Strep Nterm 2 FW | GGAGGTTCGGGAGGTTCGTTGGATTTCGGGCTGTTTCCG |
| 34. | Rem Str EccC5xen FW | CCGACCTGCGCCGGTTTAAATAGCTTAAGGCAGGCACCTCTTGAG |
| 35 | Rem Str EccC5xen RV | CTCAAGAGGTGCCTGCCTTAAGCTATTTAAACCGGCGCAGGTCGG |