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Species-specific secretion of ESX-5 type VII substrates is determined by the linker 2 of $EccC_5$

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Abstract

Mycobacteria use type VII secretion systems (T7SSs) to translocate a wide range of proteins across their diderm cell envelope. These systems, also called ESX systems, are crucial for the viability and/or virulence of mycobacterial pathogens, including *Mycobacterium tuberculosis* and the fish pathogen *Mycobacterium marinum*. We have previously shown that the *M. tuberculosis* ESX-5 system is unable to fully complement secretion in an *M. marinum esx-5* mutant, suggesting species specificity in secretion. In this study, we elaborated on this observation and established that the membrane ATPase $EccC_5$, possessing four (putative) nucleotide-binding domains (NBDs), is responsible for this. By creating *M. marinum*-*M. tuberculosis* $EccC_5$ chimeras, we observed both in *M. marinum* and in *M. tuberculosis* that secretion specificity of PE_PGRS proteins depends on the presence of the cognate linker 2 domain of $EccC_5$. This region connects NBD1 and NBD2 of $EccC_5$ and is responsible for keeping NBD1 in an inhibited state. Notably, the ESX-5 substrate EsxN, predicted to bind to NBD3 on $EccC_5$, showed a distinct secretion profile. These results indicate that linker 2 is involved in species-specific substrate recognition and might therefore be an additional substrate recognition site of $EccC_5$.

KEYWORDS

chimeras, ESX, membrane ATPase, mycobacterium, substrate specificity, type VII secretion

1 | INTRODUCTION

Type VII secretion systems (T7SSs) are crucial virulence determinants for pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium marinum* (Groschel *et al*., 2016). Pathogenic mycobacteria can have up to five T7SSs, named ESX-1 to ESX-5 (Houben *et al*., 2014), of which three, i.e., ESX-1, ESX-3 and ESX-5, have been shown to be functional (Pym *et al*., 2002; Stanley *et al*., 2003; Abdallah *et al*., 2006; Siegrist *et al*., 2009; Simeone *et al*., 2012). These secretion systems are paramount for diverse processes, such as the utilization of nutrients and iron, and completion of the macrophage infection cycle. In pathogenic mycobacteria, ESX-1 is crucial for intracellular survival by mediating phagosomal membrane rupture (van der Wel *et al*., 2007; Simeone *et al*., 2012). The importance of this secretion system is further substantiated by the fact that the lack of a large part of the *esx-1* gene cluster is the decisive factor in the attenuation of the live vaccine strain *Mycobacterium bovis* BCG (Pym *et al*., 2002; Simeone *et al*., 2012). Both ESX-3 and ESX-5 systems are essential for in vitro growth and have been linked to iron and fatty acid uptake respectively (Serafini *et al*., 2009; Siegrist *et al*., 2009; Ates *et al*., 2015).

In mycobacteria, T7SSs secrete a diverse array of substrates, which includes monomeric as well as heterodimeric protein pairs.

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Most well known are the Esx substrates, which are small proteins forming heterodimeric complexes (Renshaw *et al.*, 2005). The first described proteins of this family are the ESX-1 substrate EsxA (also named ESAT-6) and its secretion partner EsxB (also called CFP-10). Dimerization of Esx proteins is mediated by two helix-turn-helix structures and in mycobacteria one of the partner proteins harbors a C-terminal conserved secretion motif YxxxD/E (Poulsen *et al.*, 2014). Two major classes of other T7SS substrates, called the PE and PPE proteins, also form stable heterodimers (Strong *et al.*, 2006; Chen *et al.*, 2017). The PE proteins, named after a conserved proline (P) and glutamic acid (E) motif located N-terminally, have a conserved N-terminal domain of approximately 110 residues. This domain forms a helix-turn-helix structure, followed by an YxxxD/E motif, similar to Esx proteins (Strong *et al.*, 2006; Daleke, *et al.*, 2012a; Chen *et al.*, 2017). PPE proteins, named after a similar conserved motif with an additional proline (P) residue, have a larger conserved N-terminal domain of ~180 amino acids. Part of this conserved domain forms a helix-turn-helix structure involved in the dimerization of the PPE with its PE partner (Strong *et al.*, 2006; Chen *et al.*, 2017). The N-terminal PPE domain furthermore contains a so-called helical-tip domain that does not interact with the partner protein (Strong *et al.*, 2006; Chen *et al.*, 2017). Both PE and PPE proteins can have additional C-termini that are highly variable and might make up the functional domain of the substrate (Mishra *et al.*, 2008; Daleke *et al.*, 2011; Burggraaf *et al.*, 2019). The majority of the PE and PPE proteins are secreted by the ESX-5 system (Abdallah *et al.*, 2009; Ates *et al.*, 2015). ESX-5 is the most recently evolved mycobacterial T7SS and is present only in the so-called slow-growing mycobacteria, which includes important pathogens such as *M. tuberculosis* and *Mycobacterium leprae*. A large portion of the substrates that are secreted by the ESX-5 system belong to the subfamily of the PE_PGRS proteins, named after the polymorphic GC-rich repetitive sequence motifs in their genes. Although this subfamily contains many members in *M. tuberculosis*, their function is not very clear. The *M. tuberculosis* PE_PGRS30 protein has been shown to be involved in virulence (Bottai *et al.*, 2012; Iantomasi *et al.*, 2012; Fishbein *et al.*, 2015; Deng *et al.*, 2017) and PE_PGRS33 has been shown to interact with TLR2 (Basu *et al.*, 2007). However, a recent study implicated that a *M. tuberculosis* strain not secreting any PE_PGRS proteins due to a spontaneous *ppe38* deletion showed in fact (moderate) hypervirulence at later time points (Ates *et al.*, 2018). Due to the same mutation this strain was also deficient in the secretion of the so-called PPE_MPTR proteins.

The different mycobacterial T7SSs contain a set of conserved components, including two cytosolic and five membrane proteins. The cytosolic chaperone EspG has been shown to be involved in substrate recognition (Daleke *et al.*, 2012b; Ekiert and Cox, 2014; Korotkova *et al.*, 2014; Phan *et al.*, 2017). EspG interacts specifically with PE/PPE heterodimers and helps to keep these dimers soluble by binding to a hydrophobic patch on the helical-tip domain of the PPE protein (Daleke *et al.*, 2012b; Ekiert and Cox, 2014; Korotkova *et al.*, 2014). By swapping the helical-tip domain of PPE substrates of different systems, these substrates can be redirected from one system to another (Phan *et al.*, 2017), showing that this domain is involved in determining system specificity. Four of the conserved membrane components (EccBCDE) assemble into a hexameric complex of approximately 2 MDa (Houben *et al.*, 2012). While a first low-resolution image of a full T7SS membrane complex, i.e., of a hexameric ESX-5 membrane complex, has been provided by negative stain electron microscopy (EM) (Beckham *et al.*, 2017), very recently two high-resolution structures of a dimeric complex of ESX-3 have been solved by cryo-EM (Famelis *et al.*, 2019; Poweleit *et al.*, 2019). Although the quaternary structures differ between the ESX-3 and ESX-5 complex, ESX-3 has also been observed to form higher order multimers, suggesting the dimeric structure is a subcomplex (Famelis *et al.*, 2019; Poweleit *et al.*, 2019). Another discrepancy between the negative stain structure and the cryo-EM structures is the reported EccBCDE stoichiometry of 1:1:1:1 and 1:1:2:1 respectively. This variation might be due to the highly hydrophobic and thereby aggregation-prone nature of EccD, which could result in its underrepresentation in stoichiometric measurements (Beckham *et al.*, 2017; Famelis *et al.*, 2019). The fifth conserved and essential membrane component, the subtilisin-like protease mycosin or MycP, interacts only transiently with this complex and is involved in complex stabilization (van Winden *et al.*, 2016). This component is also involved in cleaving specific substrates (Ohol *et al.*, 2010).

A central component of T7SS is EccC, which is a membrane-associated ATPase and most likely the motor protein of the membrane complex (Houben *et al.*, 2012). Importantly, EccC is the only conserved membrane protein in the more distantly related T7SSs of *Firmicutes* (Abdallah *et al.*, 2007). The ATPase contains two predicted N-terminal transmembrane domains, three nucleotide binding domains (NBDs) and an extra flexible domain of unknown function (DUF) between the ATPase and transmembrane domains (Figure 1b,c) (Rosenberg *et al.*, 2015; Beckham *et al.*, 2017). The recent cryo-EM structure of ESX-3 revealed that the DUF domain exhibits an ATPase fold, similar to the previously described NBDs of EccC (Rosenberg *et al.*, 2015; Famelis *et al.*, 2019; Poweleit *et al.*, 2019). All three NBDs as well as the DUF domain of EccC are part of a family of so-called P-loop NTPases that show strong similarities to FtsK/SpoIIIE proteins. Proteins in this family use the energy released from ATP hydrolysis to drive the translocation of macromolecules (Burton and Dubnau, 2010). Whereas the activity of NBD2 and NBD3 of EccC has been shown to be partially dispensable for secretion, it is NBD1, normally held in an auto-inhibited state, that is crucial for T7SS activity (Ates *et al.*, 2015; Rosenberg *et al.*, 2015). The EccC protein of the ESX-1 system has the unique feature that it is split up in two subunits, i.e., $EccC_{a1}$ and $EccC_{b1}$. The C-terminal 7 amino acids of EsxB have been shown by yeast-two-hybrid and pull-down analysis to interact with the $EccC_{b1}$ subunit (Champion *et al.*, 2006; Rosenberg *et al.*, 2015). Subsequently, structural analyses of co-crystals of EccC from *Thermonospora curvata* (Rosenberg *et al.*, 2015) and NBD3 of $EccC_{b1}$ of *M. tuberculosis* (Wang *et al.*, 2019) with a peptide mimicking the C-terminal domain of cognate EsxB and full-length *M. tuberculosis* EsxB, respectively, revealed again that the last seven amino acids of the peptide were bound to NBD3 of EccC.

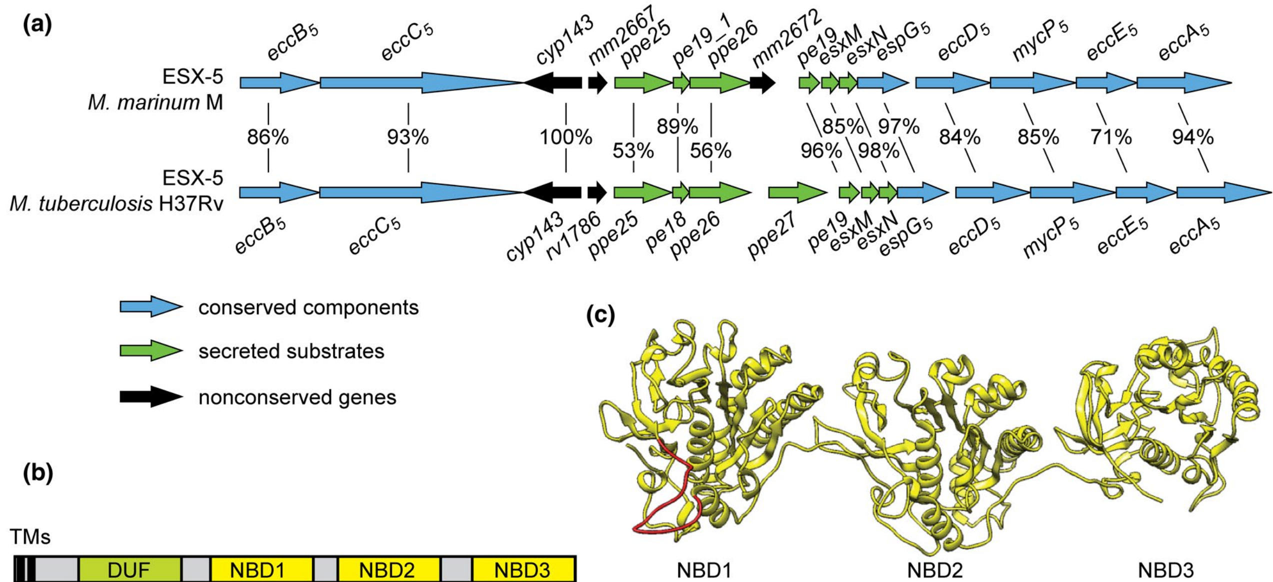

FIGURE 1 (a) Genetic organization of the *esx-5* loci in *M. marinum* and in *M. tuberculosis*. The shared identities of the orthologous proteins encoded by the genes are indicated. (b) General domain architecture of mycobacterial EccC ATPases. The constructs contain: TM, transmembrane domains; DUF, domain of unknown function, shown to adopt an ATPase fold for $EccC_3$ (Famelis *et al.*, 2019; Poweleit *et al.*, 2019); NBD, nucleotide binding domain. (c) Structural model of the *M. marinum* $EccC_5$ ATPase domains (residues 432–1388), generated with Phyre2 using the structure of *T. curvata* EccC as a template. The missing 65 residues of the linker 2 region that could not be modeled are indicated in red

However, substrate binding to EccC has only been shown for Esx proteins and it therefore remains unclear whether and how the other substrate classes, in particular the major substrate group of PE and PPE proteins, bind to this membrane ATPase. In addition, to what extent EccC is able to recognize substrates in a system-specific fashion is still very poorly understood.

Previous results from our group have shown that the ESX-5 system is essential for growth of the fish pathogen *M. marinum* (Ates *et al.*, 2015). Strikingly, this essentiality can be circumvented by increasing the permeability of the mycobacterial outer membrane, e.g., by introducing an outer membrane porin from *Mycobacterium smegmatis*, called MspA (Ates *et al.*, 2015). Expression of the homologous *esx-5* operon from *M. tuberculosis* also allowed for the successful deletion of the entire operon in *M. marinum* (Ates *et al.*, 2015). Although the ESX-5 system of *M. tuberculosis* is able to mediate growth of the *M. marinum* Δ*esx-5* mutant, the complementation is only partial as the *M. tuberculosis* system is unable to mediate the secretion of many *M. marinum* ESX-5 substrates, most of which are *M. marinum*-specific PE_PGRS proteins (Ates *et al.*, 2015). This result was unexpected, because the components of the two systems have an overall amino acid identity of 78% (Figure 1a). We speculated that the observed secretion defects are caused by the fact that many *M. marinum* substrates are not recognized by the *M. tuberculosis* ESX-5 system. If this assumption is correct, we expected that the responsible proteins could be the two conserved components shown to be involved in substrate recognition, i.e., $EspG_5$ and $EccC_5$. In this study, we tested this hypothesis by investigating the species-specific roles of $EspG_5$ and $EccC_5$ in ESX-5-mediated secretion.

2 | RESULTS

2.1 | $EspG_{5mtub}$ complements secretion of an *M. marinum* Δ$espG_5$ mutant

EspG is a dedicated T7SS chaperone present in four of the five ESX systems, which binds PE/PPE proteins in a system-specific fashion (Daleke *et al.*, 2012b; Korotkova *et al.*, 2014). As we hypothesized that the inability of the *M. marinum* Δ*esx-5*::$esx\text{-}5_{mtub}$ to secrete most *M. marinum* PE/PPE substrates is due to the species-specific recognition of these substrates, $EspG_5$ was a prime candidate for causing this effect. To test this, we used an *M. marinum* Δ$espG_5$ knock-out strain that expresses MspA to circumvent the essentiality of ESX-5 for growth (Phan *et al.*, 2018). As previously demonstrated, this mutant showed no PE_PGRS secretion, as determined by analyzing cell surface fractions extracted by the mild detergent Genapol X-080 (Figure S1). Also the ESX-5 substrate EsxN was not secreted by this mutant (Figure S1). This reduction in secretion in the $espG_5$ mutant did not result in increased amounts of ESX-5 substrates in the cell, which is consistent with previous observations made for *esx-5* mutants in *M. marinum* (Houben *et al.*, 2012; Ates *et al.*, 2015; Ates *et al.*, 2016; Ates *et al.*, 2018; van Winden *et al.*, 2019). In these previous studies, it was shown that this instability phenotype is not linked to regulatory effects on a transcriptional level (Abdallah *et al.*, 2009; Bottai *et al.*, 2012; Houben *et al.*, 2012; Ates *et al.*, 2018). PE_PGRS protein secretion could be restored to WT levels upon complementation with the *M. marinum* $espG_5$ gene, although EsxN secretion was only partially restored (Figure S1). Importantly, introduction of

the *M. tuberculosis* $espG_5$ gene restored secretion to similar levels (Figure S1). We therefore conclude that $EspG_{5mtub}$ is fully functional in *M. marinum* and not the cause for the *M. marinum* Δ*esx-5*::*esx-5*$_{mtub}$ species-specific secretion defect of PE_PGRS proteins.

2.2 | $EccC_{5mtub}$ complements essentiality but not PE_PGRS secretion in an *M. marinum* Δ$eccC_5$ mutant

The central membrane ATPase $EccC_5$ is the second T7SS protein that has been shown to bind substrates (Stanley *et al.*, 2003; Rosenberg *et al.*, 2015; Wang *et al.*, 2019). We therefore examined whether EccC was responsible for species-specific secretion. First, we checked whether $eccC_{5mtub}$ was able to rescue the essentiality phenotype of an *M. marinum* Δ$eccC_5$ strain by introducing the same plasmid into a previously described Δ$eccC_5$::$eccC_{5mmar}$ strain that lacks MspA (Ates *et al.*, 2015). This strain bears an integrative plasmid containing both $eccC_{5mmar}$ and a hygromycin resistance marker, which was exchanged with a kanamycin-resistant integrative plasmid harboring $eccC_{5mtub}$. Multiple colonies appeared that showed kanamycin resistance and hygromycin sensitivity, demonstrating that the plasmid exchange was successful and therefore that $eccC_{5mtub}$ is able to mediate growth of the *M. marinum* Δ$eccC_5$ mutant.

Next, we tested the secretion profile of an *M. marinum* Δ$eccC_5$ mutant expressing MspA, which was complemented with $eccC_5$ from either *M. marinum* or *M. tuberculosis*. As expected, this MspA-expressing Δ$eccC_5$ mutant showed no secretion of PE_PGRS or EsxN substrates (Figure 2c). While secretion could be restored to WT levels upon complementation with the *M. marinum* $eccC_5$ gene, introduction of a plasmid containing $eccC_5$ from *M. tuberculosis* showed no visible PE_PGRS proteins in the cell surface-enriched protein fraction (Figure 2c). Surprisingly, the culture supernatant fraction of this strain showed only one PE_PGRS protein band, which was similar to the previously observed PE_PGRS secretion phenotype of *M. tuberculosis* (Figures 2c and 3a) (Houben *et al.*, 2012). In addition, no EsxN secretion was observed in this strain. The difference in secretion is surprising, as the overall sequence identity between the two $eccC_5$ orthologues is 93% (Figure 1a). In addition, the residues present in the pocket of NBD3, which have been predicted to be crucial for Esx substrate binding (Rosenberg *et al.*, 2015; Wang *et al.*, 2019) are conserved between the two species. On the other hand, the most C-terminal residues of EsxM, the partner protein of EsxN, containing the C-terminal YxxxD/E motif and predicted to be involved in EccC binding, are not well conserved between *M. marinum* and *M. tuberculosis* (Figure S4b). We also checked the colony phenotype for the mutant and the complemented strains. Opposed to the WT and the Δ$eccC_5$::$eccC_{5mmar}$ strain, which showed smooth colony morphology and monodispersed growth in liquid cultures, the Δ$eccC_5$ as well as the Δ$eccC_5$::$eccC_{5mtub}$ strains showed a nondispersed growth phenotype in culture and flat and dry colonies on plate (Figure S2). Together, these results show that $eccC_{5mtub}$ is unable to fully complement the $eccC_5$ mutation in *M. marinum* due to species-specific functioning.

We next investigated whether the observed secretion defects were due to the unsuccessful incorporation of $EccC_{5mtub}$ in the ESX-5 membrane complex. We have shown previously that the ESX-5 membrane complex of ~2 MDa can be visualized using BN-PAGE and western blot analysis of DDM solubilized cell envelopes using antibodies against any of the four complex components (Houben *et al.*, 2012). Using the same approach, the Δ$eccC_5$ strain showed reduced expression of the ESX-5 membrane components $EccB_5$ and $EccE_5$ and membrane complex formation was abrogated (Figure S3a). Complex formation was restored upon complementation with either the *M. marinum* or *M. tuberculosis* $eccC_5$ containing plasmid (Figure S3a). Similarly, expression of the $EccB_5$ and $EccE_5$ components were restored to WT levels (Figure S3b). We therefore conclude that the lack of PE_PGRS secretion by the *M. marinum* Δ$eccC_5$::$eccC_{5mtub}$ was probably not caused by any defect in the assembly of the ESX-5 membrane complex. Because the secretion phenotype of the Δ$eccC_5$::$eccC_{5mtub}$ strain was similar to that of the Δ*esx-5*::*esx-5*$_{mtub}$ complementation strain, we conclude that EccC is the key component responsible for this distinct secretion defect.

2.3 | $EccC_5$ linker 2 domain is involved in species-specific secretion in *M. marinum*

Although $EccC_{5mtub}$ is properly integrated in the ESX-5 membrane complex and rescues essentiality of an *M. marinum* Δ$eccC_5$ mutant, this ATPase was unable to restore secretion of all substrates. A sequence alignment of the $EccC_5$ proteins of *M. marinum* and *M. tuberculosis* showed high overall conservation, but also revealed some variations (Figures S4a and S6). Aligning the two proteins with the sequence of the *T. curvata* EccC, for which the crystal structure has been solved (Rosenberg *et al.*, 2015), revealed that the amino acids that were shown to be important for ATPase activity and substrate binding are highly conserved (Figures S4a and S6). In particular, the interacting amino acids lining the substrate-binding pocket on NBD3, i.e., E1237, L1253, I1282 for $eccC_{5mtub}$ and E1234, L1250, I1279 for $eccC_{5mmar}$ (I1163, I1179 and L1208 in the *T. curvata* system), are all conserved (Figure S6). In addition, also critical residues within the linker 2 region are conserved, i.e., the tryptophan ($W810_{mtub}$ and $W807_{mmar}$) and glutamine ($Q811_{mtub}$ and $Q808_{mmar}$ (L763 in *T. curvata*)) that keep the critical first NBD in an inhibited state (Rosenberg *et al.*, 2015). However, there are key differences in the same linker 2 region (Figure S4a). As this domain blocks ATPase activity of NBD1, it has been speculated that a, yet unknown, event might lead to the displacement of linker 2 from the pocket of NBD1, allosterically regulating its activity (Rosenberg *et al.*, 2015). Interestingly, a significant part (41 residues) of this linker 2 domain is disordered and therefore not present in the crystal structure of EccC of *T. curvata* (Figure 2a). This disordered region also revealed the lowest sequence identity between the EccC proteins of *M. tuberculosis* and *M. marinum*. Compared to EccC of *T. curvata*, the linker 2 region is considerably larger for the $EccC_5$ proteins, with an additional 31 residues for $EccC_{5mtub}$ and 27 residues for $EccC_{5mmar}$.

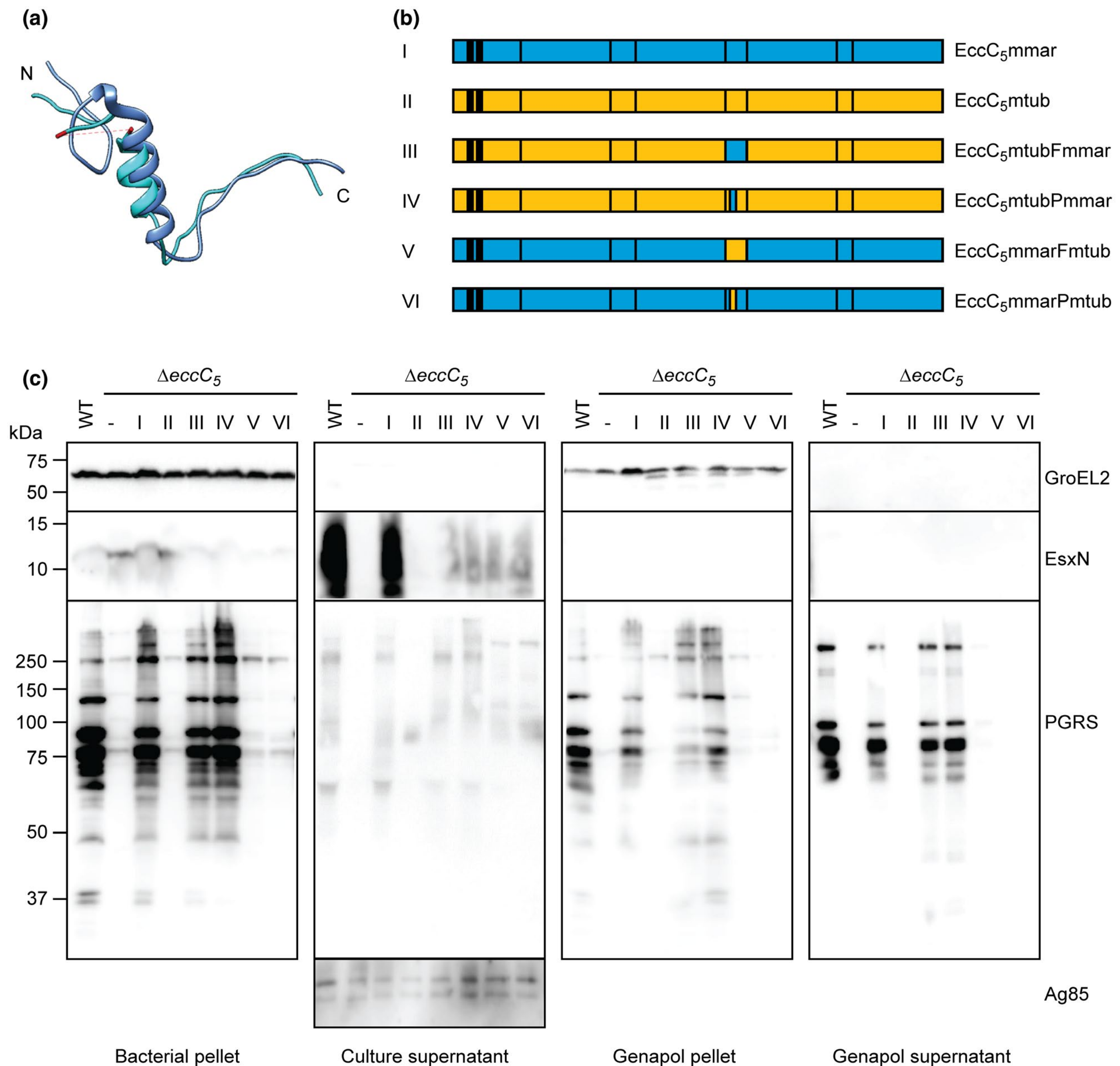

FIGURE 2 Role of linker 2 in substrate specificity in *M. marinum* ESX-5. (a) Superimposition of *T. curvata* linker 2 (bright blue) and linker 3 (dark blue). The 43 residues that were not resolved in the crystal structure are indicated in red. (b) Schematic overview of *M. marinum* and *M. tuberculosis* $EccC_5$ as well as the chimeric constructs used to complement $eccC_5$ mutants of these species. See Figure S4a for the exchanged sequences. (c) Secretion analysis of *M. marinum* $\Delta eccC_5$ complemented with WT $eccC_{5mmar}$, $eccC_{5mtub}$ and chimeric constructs depicted in b. Proteins were visualized by SDS-PAGE and immunoblotting using antibodies against EsxN and PE_PGRS proteins (ESX-5 substrates), GroEL2 (lysis and whole-cell loading control) and Ag85 (secreted fraction loading control)

Taking these observations into consideration, we reasoned that this disordered region in linker 2 might play a crucial role in the (in) activation of NBD1 through regulating substrate binding and/or specificity. In order to test this, we made two chimeric $eccC_5$ constructs where the backbone originates from *M. tuberculosis* and the linker 2 region from *M. marinum*. The linker 2 portion covered either the entire region after NBD1 until just after the two amino acids WQ that interact with the pocket of NBD1 (named full—$EccC_{5mtub}F_{mmar}$) or only a small part of the linker 2 that shows the most sequence divergence between the two and also aligns broadly with the disordered region (named partial—$EccC_{5mtub}P_{mmar}$) (Figures 2b and S4A). Both constructs could rescue the essentiality of the *M. marinum* $eccC_5$ knockout in the absence of MspA. Subsequently, we examined the expression of ESX-5 membrane components and the presence of the ESX-5 membrane complex by SDS- and BN-PAGE immunoblot analysis, which showed that both proteins were incorporated (Figure S3a,b). Finally, while expression of the original $EccC_{5mtub}$ in the $eccC_5$ mutant resulted in flat and dry colonies, this phenotype was reversed to the WT situation upon exchange of the linker 2 in the $EccC_{5mtub}F_{mmar}$ or $EccC_{5mtub}P_{mmar}$ plasmids (Figure S2).

We next checked if these constructs could alleviate the secretion defect caused by the $EccC_{5mtub}$ complementation. Remarkably,

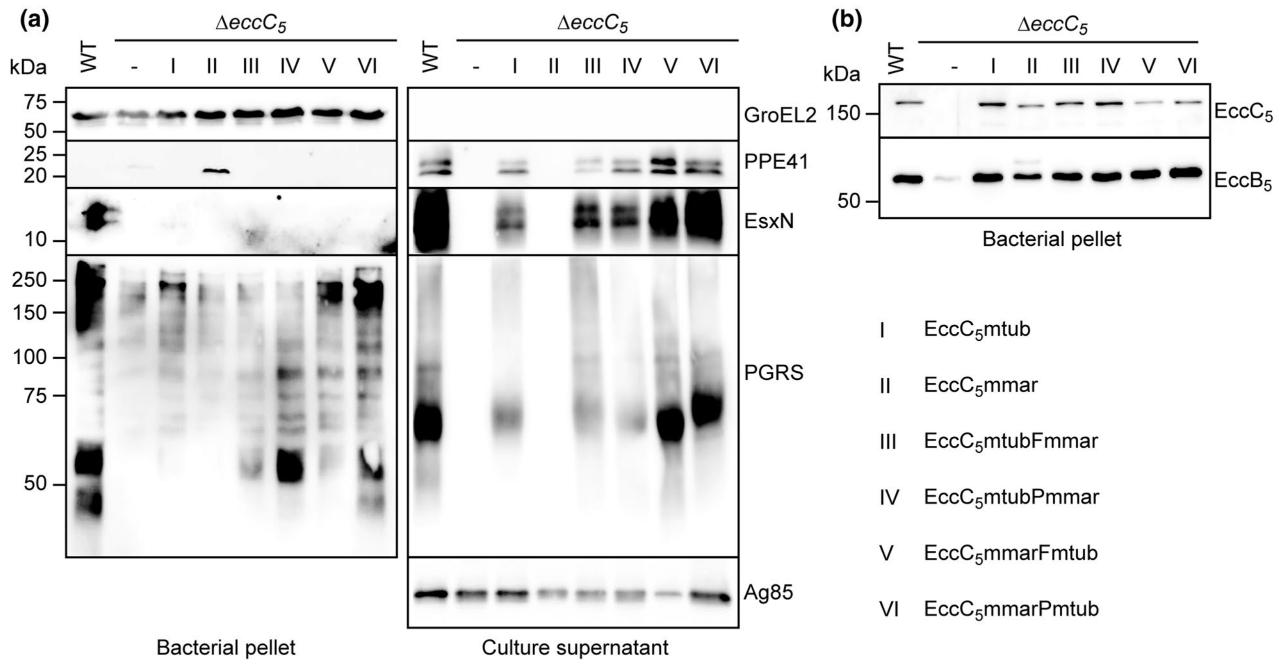

FIGURE 3 Role of linker 2 in substrate-specificity in *M. tuberculosis* ESX-5. (a) Secretion analysis of a *M. tuberculosis* $EccC_5$ transposon mutant complemented with WT $eccC_{5mmar}$, $eccC_{5mtub}$ and chimeric constructs. Roman numerals indicate the constructs as depicted on the bottom right (see Figure 2b for a schematic overview of the $EccC_5$ proteins). Proteins were visualized by SDS-PAGE and immunoblotting using antibodies against EsxN, PE_PGRS proteins and PPE41 (ESX-5 substrates), GroEL2 (lysis and whole-cell loading control) and Ag85 (secreted fraction loading control). (b) SDS-PAGE and immunoblot analysis of whole cells of *M. tuberculosis* WT, $\Delta eccC_5$ and $\Delta eccC_5$ complemented with constructs depicted in Figure 2b. Proteins were visualized using antibodies against $EccB_5$ and $EccC_5$ (membrane components of ESX-5)

although EsxN was not fully restored, secretion of PE_PGRS proteins was restored back to WT levels with both full and partial swapped constructs (Figure 2c). This is intriguing, as only 19 amino acids were different between the swapped region in the partial construct and the WT $eccC_{5mtub}$ gene. These data confirmed our hypothesis that linker 2 is involved in substrate specificity and/or (in)activation of NBD1.

Subsequently, we wondered if conversely, we could repress secretion of the $\Delta eccC_{5mmar}$::$eccC_{5mmar}$ complementation by swapping the linker 2 of $EccC_{5mmar}$ with that of $EccC_{5mtub}$. Importantly, although the linker 2 region originated from $EccC_{5mtub}$, the rest of the gene was *M. marinum* WT, thus keeping all other (un)known potential interaction sites. These constructs were named $EccC_{5mmar}F_{mtub}$ and $EccC_{5mmar}P_{mtub}$ (Figures 2b and S4a). Although only 25 residues for the full linker 2 and 19 residues for partial linker 2 were different, PE_PGRS secretion with the chimeric constructs was completely abolished, thus substantiating our initial findings. Conversely, EsxN was present in the supernatant, but only in low amounts, similar to the reciprocal chimeric constructs. Both constructs could rescue the essentiality of the *M. marinum* $\Delta eccC_5$ mutant and showed a somewhat intermediate phenotype between the smooth WT colonies and the rough and dry colonies of the $\Delta eccC_5$ and $\Delta eccC_5$::$eccC_{5mtub}$ (Figure S2). From this, we conclude that the linker 2 domain of $EccC_5$ is involved in species-specific secretion of PE_PGRS substrates. In addition, as EsxN secretion was only partially recovered by all chimeric constructs, the optimal secretion of EsxN is not only dependent on NBD3 but is regulated by multiple domains of or interactions with EccC.

2.4 | $EccC_5$ linker 2 is involved in substrate specificity in *M. tuberculosis*

We next tested whether the drastic effects upon exchanging only a small part of the linker 2 of $EccC_5$, observed in *M. marinum*, could also apply to other species. For this, we introduced the same chimeric constructs as analyzed in *M. marinum* in a previously described *M. tuberculosis* $eccC_5$ transposon mutant (Figures 2b and S4a) (Houben *et al.*, 2012).

The different constructs were efficiently expressed in the *M. tuberculosis* mutant strain (Figure 3b). In addition, while expression of $EccB_5$ was strongly affected in the mutant strain, its expression was restored to WT levels in the presence of all constructs. From this, we conclude that the chimeric EccC proteins were again able to stabilize other components of the ESX-5 membrane complex, suggesting these constructs were properly integrated in the membrane complex (Figure 3b). Next, the secretion of PE_PGRS proteins and EsxN was analyzed. It should be noted that surface-associated PE_PGRS proteins are not extractable in *M. tuberculosis* (Houben *et al.*, 2012; Ates *et al.*, 2018), while in our *M. marinum* experiments only this subset of substrates was completely dependent on the cognate linker 2. Similar to *M. marinum*, in *M. tuberculosis* secretion of PE_PGRS

proteins and EsxN into the culture supernatant was completely abolished in the absence of *eccC*$_5$, and this could be restored by the WT *M. tuberculosis* gene, albeit to a slightly lower level (Figure 3a). Importantly, the *M. marinum* gene was not able to complement both PE_PGRS and EsxN secretion, identical to the reciprocal experiment in *M. marinum*. Both chimeric EccC$_5$ proteins, i.e., *M. tuberculosis* EccC$_5$ with the linker 2 of the *M. marinum* protein and the *M. marinum* protein with the linker 2 of *M. tuberculosis*, showed restored PE_PGRS and EsxN secretion, again similar to what was observed for the culture supernatant fractions of *M. marinum* (Figure 3a). From this, we conclude that the linker 2 domain has a similar role in *M. tuberculosis*, as described for *M. marinum* ESX-5.

3 | DISCUSSION

ESX-5 is the most recently evolved T7SS in mycobacteria and is responsible for the secretion of the majority of PE and PPE proteins, among which are most or even all members of the large family of PE_PGRS proteins. Previous studies have shown that introduction of the ESX-5 system of *M. tuberculosis* is able to take over the essential role of the ESX-5 system in *M. marinum* (Ates *et al*., 2015). However, this system is only marginally able to restore secretion of *M. marinum* ESX-5-dependent substrates, suggesting that substrate recognition is at least partially species specific. This study revealed that a highly specific domain in the central membrane ATPase of the ESX-5 system is the determining factor for the specifies-specific secretion of PE_PGRS proteins.

To identify the component responsible for the observed species-specific secretion of PE_PGRS proteins, we used individual *esx-5* component mutants and complemented these with the corresponding gene from either *M. marinum* or *M. tuberculosis*. Because PE_PGRS proteins were not secreted by the *M. marinum* Δ*esx-5::esx-5*$_{mtub}$ and have been widely used as model ESX-5 substrates, we decided to use these proteins as a readout for ESX-5 functionality. Additionally, by assessing PE_PGRS protein secretion, we could monitor a whole set of substrates and not just individual proteins. Notably, blocking ESX-5 secretion also leads to lower cellular levels of PE_PGRS proteins in *M. marinum*, which has been observed previously for more *esx-5* mutants, even when specific substrates were controlled by constitutive promoters (Abdallah *et al*., 2009; Ates *et al*., 2018).

EspG was our initial most prominent candidate, as this chaperone has been shown to bind PE/PPE heterodimers and the corresponding EspG-binding domain is a determining factor in the system-specific secretion of these substrates. However, Δ*espG*$_5$ complementations did not show any marked differences in secretion between EspG$_5$ of *M. marinum* and *M. tuberculosis*. As the conservation between the two proteins is very high, i.e., 97% identity (Figure 1a), both of them can probably serve each other's function in the opposite species.

The only other component known to recognize substrates is EccC, although only Esx substrates have been shown to interact with this central ATPase component (Stanley *et al*., 2003; Rosenberg *et al*., 2015; Wang *et al*., 2019). Indeed, complementation of *M. marinum* Δ*eccC*$_5$ with *eccC*$_{5mtub}$ was able to restore growth, but not the presence of PE_PGRS proteins on the cell surface or EsxN secretion in the culture supernatant. Importantly, using *M. marinum*-*M. tuberculosis* EccC$_5$ chimeras, we showed that surface-localization of PE_PGRS proteins strictly depends on the presence of the native linker 2 domain, irrespective of the origin of the EccC$_5$ backbone. The absence of surface-localized PE_PGRS proteins is also linked to a distinct flat and dry colony morphology. Interestingly, while the presence of EccC$_{5mtub}$ showed only a single PE_PGRS protein in the secreted fraction by western blotting, *M. marinum* EccC$_5$ with linker 2 of *M. tuberculosis* was able to mediate secretion of multiple PE_PGRS proteins into the culture supernatant, although the PE_PGRS protein pattern was distinct from that of the WT strain.

Significantly, we obtained similar results in *M. tuberculosis*. The only problem in studying this process in *M. tuberculosis* is that surface-associated PE_PGRS proteins are not extractable in this species (Houben *et al*., 2012; Ates *et al*., 2018). Analysis of the PE_PGRS substrates in the secreted fraction, however, shows an identical profile as in *M. marinum*. Whereas secretion analysis of an *eccC*$_5$ mutant showed abolished secretion of PE_PGRS and EsxN in both species, complementation with the native gene restores this back to WT levels. Importantly, introduction of a plasmid containing *eccC*$_5$ from the opposite species was unable to restore secretion into the culture supernatant, while expression of an EccC$_5$ chimera, which contains the native linker 2 and the remainder of the protein from the opposite species does recover secretion in both *M. marinum* and *M. tuberculosis*. This further strengthens the hypothesis that the EccC$_5$ linker 2 domain is involved in determining substrate-specificity for the secretion of PE_PGRS substrates.

Although the interface between NBD1 and NBD2 is similar to that between NBD2 and NBD3, the interdomain linkers are variable. Both these linkers form a main α-helix that potentially mimics the C-terminal tail of EsxB-like proteins, which binds to NBD3. However, there is a region of variability between linkers 2 and 3 in sequence and size immediately N-terminal from of this α-helix, i.e., this region is significantly larger in the linker 2 interdomain (Figure S5). Highly intriguing is that a large part of this variable region of linker 2 is disordered in the only available structure containing all three NBDs of an EccC homologue from *T. curvata* (Rosenberg *et al*., 2015), suggesting flexibility (Figure 2a). Sequence alignments show that this is also the most variable region between the two EccC$_5$ proteins of *M. tuberculosis* and *M. marinum* (Figure S6). An alignment of the EccC ATPases from all five mycobacterial ESX systems shows that this region of the linker 2 domain is extended in ESX-2 and ESX-5 systems, i.e., the most recently evolved systems (Figure S7). ESX-5 most likely evolved through a duplication of the *esx-2* cluster (Gey van Pittius *et al*., 2006). This duplication event is followed by the vast expansion of *pe* and *ppe* genes, in particular the most recently evolved PE_PGRS proteins and the so-called PPE_MPTR proteins, of which at least a major portion are secreted through the ESX-5 system (Abdallah *et al*., 2009; Bottai *et al*., 2012). Previous mass spectrometry analysis of an Δ*esx-5::esx-5*$_{mtub}$

strain showed that majority of PE/PPE proteins, that are not secreted by this strain, are specific for *M. marinum* (Ates *et al.*, 2015). In contrast, different ESX-5-dependent PE/PPE heterodimers that are conserved between *M. tuberculosis* and *M. marinum* showed no notable secretion difference in *M. marinum* Δ$eccC_5$::$eccC_{5mmar}$ and Δ$eccC_5$::$eccC_{5mtub}$ (our unpublished results). These findings indicate that PE/PPE proteins that are more recently evolved in *M. marinum* are not recognized by (the linker 2 of) $EccC_{5mtub}$. Therefore, the variable extension of the linker 2 domain might have co-evolved with the expansion of PE/PPE proteins, especially the PE_PGRS proteins, to allow the recognition of this vast group of substrates.

Importantly, the secretion of EsxN is also species-specific, as both the *M. marinum* and *M. tuberculosis* $eccC_5$ mutants could only be restored to WT levels upon complementation with the cognate $eccC_5$ gene. This is in contrast to the conservation found in the putative binding pocket of Esx substrates on NBD3. Notably, EsxM, i.e., the partner protein of EsxN carrying the predicted C-terminal secretion signal, has a stop codon in *M. tuberculosis*. However, both EsxN and EsxM have multiple highly homologous paralogs in *M. marinum* and *M. tuberculosis*, which probably have redundant functions. Although there is high conservation between the different EsxM homologs from *M. marinum* and *M. tuberculosis*, the most C-terminal four amino acids are divergent. As both for the *T. curvata* EccC and $EccC_{b1}$ of *M. tuberculosis* the last seven amino acids of EsxB are involved in binding to NBD3 (Rosenberg *et al.*, 2015; Wang *et al.*, 2019), this provides an explanation for the species-specific secretion of EsxN. However, our observation that all chimeric $EccC_5$ proteins with exchanged linker 2 regions, irrespective of the origin of NBD3, showed intermediate levels of EsxN secretion in *M. marinum* and full secretion in *M. tuberculosis* is more difficult to explain. As T7SS substrates have been shown to be interdependent on each other for secretion (Fortune *et al.*, 2005; Champion *et al.*, 2009; Ates *et al.*, 2018; Damen *et al.*, 2020), it might be possible that PE/PPE protein(s) and EsxM/EsxN are secreted in a concerted fashion. Indeed, it has recently been shown that the ESX-1 secreted heterodimer EsxB_1/EsxA_1 is efficiently secreted only when PE35/PPE68_1, which are encoded by the same operon as the Esx proteins, are co-expressed and secreted (Damen *et al.*, 2020). While substrate interdependency for secretion is a not yet understood phenomenon in T7SS, these data together suggest that secretion-specificity of Esx heterodimers is not only dependent on the interaction with the cognate EccC NBD3.

Based on our current results and previous data we propose a working model for substrate recognition by EccC in mycobacterial T7SSs (Figure 4). In this model, the EccBCDE membrane complex is a stable complex in the mycobacterial inner membrane. Without substrate binding, EccC is hexameric via its transmembrane regions, while its cytosolic domain is highly flexible through the N-terminal DUF domain (Beckham *et al.*, 2017). Interaction of the C-terminal NBD3 with the C-terminal tail of specific (Esx) substrates drives multimerization, but NBD1 remains inactive through the pocket 1/linker 2 connection (Rosenberg *et al.*, 2015). The final secretion activation step takes place upon linker 2 displacement from pocket 1, which at least in the mycobacterial ESX-5 system, is triggered by binding of PE/PPE substrates or a (yet unknown) chaperone for these proteins. While the role of linker 2 of EccC in keeping the highly important NBD1 in an inactive state has previously been described, we present here a new role for this domain of EccC ATPases in substrate specificity.

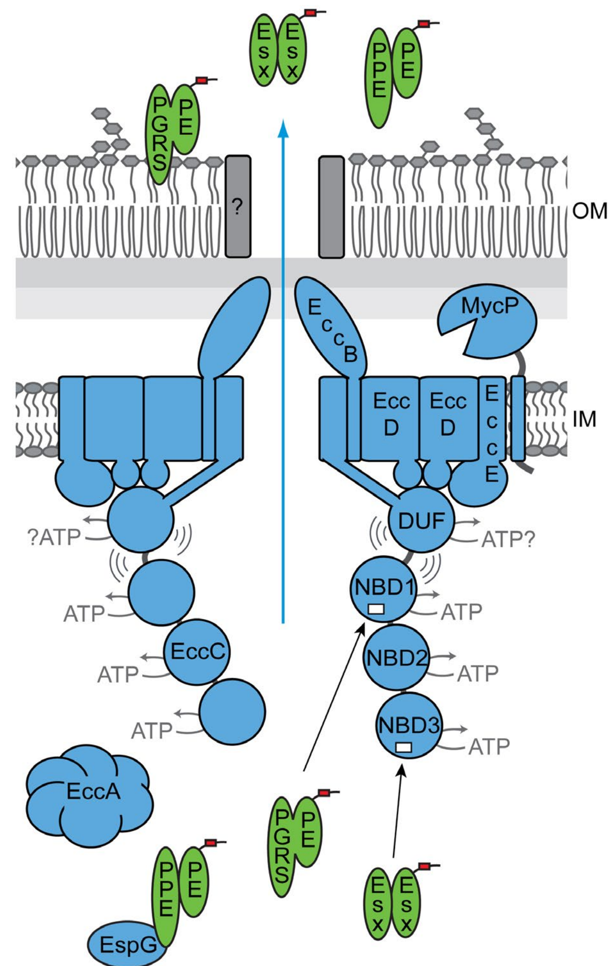

FIGURE 4 Model for ESX-5 mediated secretion. Substrate recognition occurs at two separate sites on the $EccC_5$ ATPase. The third NBD interacts with the C-terminus of specific Esx proteins, leading to the multimerization of the soluble domain of EccC. A second site of substrate recognition is located in the linker 2 domain at the first NBD. As the interaction between NBD1 and this linker has an inhibitory effect on the activity of NBD1, binding of PE_PGRS proteins and perhaps also other PE and PPE proteins disrupts this interaction and activates this crucial ATPase domain

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and culture conditions

M. marinum M and *M. tuberculosis* CDC155 were used for all experiments involving $EccC_5$ and *M. marinum* E11 was used for the $EspG_5$ analysis. *M. marinum* was grown at 30°C on 7H10 agar with 10% Middlebrook OADC (BD Biosciences) or in 7H9 liquid medium with 10% Middlebrook ADC and 0.05% Tween 80 (Merck). *M. tuberculosis*

was grown at 37°C under similar conditions. Culture medium was supplemented with the necessary antibiotics at the following concentrations: kanamycin, 25 µg/ml; hygromycin, 50 µg/ml; streptomycin, 30 µg/ml.

4.2 | Molecular cloning

All cloning was performed in *Escherichia coli* DH5α, with restriction enzymes from New English Biolabs and PCR amplifications with Iproof (BioRad). Difficult ligations or ligations that included more than two fragments were performed with In-Fusion (TakaraBio) with 15 bp homologies coded in the primer sequence. Two identical pMV361 vectors cut with XmnI and HindIII and coding for Hyg^R or Kan^R were used (Ates *et al.*, 2015). The *esxM–esxN–espG*$_5$ region or *eccC*$_5$ was amplified from *M. tuberculosis* H37Rv genomic DNA using anchored primers 1 and 2 or 3 and 4, respectively (XmnI, HindIII, Table S2), and ligated in two pMV361 vectors coding for Hyg^R or Kan^R (Ates *et al.*, 2015) using XmnI and HindIII, resulting in the plasmids pMV-*espG*$_{5mtub}$-*hyg*^{R} and pMV-*eccC5mtub-kan*^{R}. Primers 5 and 6 were used to PCR amplify the partial linker 2 domain (between F744 and V786) from the pMV-*eccC*$_{5mmar}$ plasmid. This PCR product and the pMV-*eccC*$_{5mtub}$ plasmid were both cut with FspAI and MunI and ligated, resulting in the plasmid pMV-*eccC*$_{5mtub}$*P*$_{mmar}$.

Primers 7 and 8 were used to amplify the full linker 2 domain of *eccC*$_{5mmar}$ (between residues A680 and D819) and primers 9 and 10 were used to PCR amplify the rest of the *eccC*$_{5mtub}$ gene, downstream of the linker 2, which was cut out in the process. Plasmid pMV-*eccC*$_{5mtub}$ was cut with SfiI and HindIII and ligated with both PCR products via In-Fusion cloning, resulting in plasmid pMV-*eccC*$_{5mtub}$*F*$_{mmar}$. Primers had a 15bp overlap for In-Fusion cloning.

pMV-*eccC*$_{5mmar}$*F*$_{mtub}$ and pMV-*eccC*$_{5mmar}$*P*$_{mtub}$ were cloned in a similar fashion. Due to a lack of proper restriction sites, these plasmids were cloned from scratch using In-Fusion cloning. The *eccC*$_{5mmar}$ region upstream of the linker 2 domain was amplified from the pMV-*eccC*$_{5mmar}$ plasmid with primers 11 and 12 for the full domain (PCR1) and primers 11 and 17 for the partial domain (PCR2). The *eccC*$_{5mtub}$ linker 2 domain was amplified from the corresponding plasmid with primers 13 and 14 for the full (between residues A679 and D822—PCR3) and 18 and 19 for the partial domain (between residues F743 and T789—PCR4). The *eccC*$_{5mmar}$ region downstream of the linker 2 domain was PCR amplified from pMV-*eccC*$_{5mmar}$ with primers 15 and 16 for the full domain—PCR5—and primers 16 and 20 for the partial domain—PCR6. A pMV-*kan*^{R} plasmid cut with the XmnI and HindIII restriction sites was In-Fusion ligated with PCR products 1, 3 and 5 to result in the plasmid pMV-*eccC*$_{5mmar}$*F*$_{mtub}$ and with PCR products 2, 4 and 6 to result in the plasmid pMV-*eccC*$_{5mmar}$*P*$_{mtub}$.

4.3 | Protein secretion and western blot analysis

For protein secretion, *M. marinum* strains were grown in 7H9 liquid medium with 10% Middlebrook ADC, 0.05% Tween 80 and appropriate antibiotics until mid-log phase. Cells were harvested, washed and inoculated at an OD_{600} of 0.4–0.5 in 7H9 liquid medium with 0.2% dextrose, 0.2% glycerol, 0.05% Tween 80 and appropriate antibiotics. After overnight growth, cells were pelleted at an OD_{600} of 0.8–1. Supernatants were passed through an 0.2 µm filter and precipitated with trichloroacetic acid (TCA) (culture supernatant fraction). Cell pellets were split in two and half was treated with 0.5% Genapol X-080 (Fluka) for 30 min, head over head at room temperature, after which cells were spun down and supernatant was collected (Genapol surface-extracted fraction and Genapol-treated cells). Both whole cell samples, treated or not with Genapol-X080, were lysed by bead-beating. SDS loading buffer was added and samples were boiled and loaded on SDS-PAGE gels (10%–16%, depending on the size of the proteins of interest), transferred to nitrocellulose membranes and stained with appropriate antibodies. For experiments involving *M. tuberculosis*, the procedure was similar, but cells and culture supernatants were heat inactivated for 30 min at 80°C after harvesting. The antibodies that were used were anti-GroEL2 (CS44; John Belisle, NIH, Bethesda, MD, USA), anti-EsxN (Mtb9.9a), anti-PE_PGRS (Abdallah *et al.*, 2006), anti-Ag85 (Bei Resources), anti-PPE41 (Abdallah *et al.*, 2006), anti-EspG$_5$ (Houben *et al.*, 2012), anti-EccB$_5$ (Houben *et al.*, 2012), anti-EccC$_5$ (Houben *et al.*, 2012), anti-EccE$_5$ (Houben *et al.*, 2012) and anti-FtsH (Houben *et al.*, 2012).

4.4 | Cell envelope isolation

For cell envelope isolations, *M. marinum* was grown in liquid 7H9 media with 10% Middlebrook ADC, 0.05% Tween 80 and appropriate antibiotics to an OD_{600} of 1.2–1.5. Cells were washed in PBS and resuspended in CE buffer (20 mM Tris-HCl, 300 mM NaCl and 10% glycerol). Cells were lysed by passing through a One-Shot Cell disruptor (Constant Systems Ltd.) and unbroken cells were pelleted at 5,000× *g*. Cell envelopes (CE) were separated from the soluble fraction by ultracentrifugation at 150.000× *g* for 90 min. After ultracentrifugation, supernatant was discarded, pellets were washed in CE buffer and resuspended in CE buffer.

5 | BN-PAGE

For BN-PAGE analysis of membrane complexes, cell envelopes were solubilized with 0.25% DDM for 1 hr at 4°C. Nonsolubilized material was pelleted by centrifugation at 100.000× *g* for 20 min at 4°C. NativePage 5% G-250 Sample Additive (Invitrogen) was added to the resulting supernatant fraction and samples were run on a 3%–12% NativePage Bis-Tris Protein Gel (Invitrogen). Gels were blotted to a PVDF membrane and stained with appropriate antibodies.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: C.M.B., W.B., E.N.G.H. Performed the experiments: C.M.B., R.U. Analyzed the data: C.M.B., W.B., E.N.G.H. Wrote the initial draft C.M.B. Manuscript finalization: C.M.B., W.B., E.N.G.H.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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