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Minireview

MINIREVIEW – Physiology & Biochemistry

# Bacterial secretion chaperones: the mycobacterial type VII case

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**One sentence summary:** The role of bacterial chaperones in substrate recognition and targeting in protein secretion with emphasis on type VII secretion
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## ABSTRACT

Chaperones are central players in maintaining the proteostasis in all living cells. Besides highly conserved generic chaperones that assist protein folding and assembly in the cytosol, additional more specific chaperones have evolved to ensure the successful trafficking of proteins with extra-cytoplasmic locations. Associated with the distinctive secretion systems present in bacteria, different dedicated chaperones have been described that not only keep secretory proteins in a translocation competent state, but often are also involved in substrate targeting to the specific translocation channel. Recently, a new class of such chaperones has been identified that are involved in the specific recognition of substrates transported via the type VII secretion pathway in mycobacteria. In this minireview, we provide an overview of the different bacterial chaperones with a focus on their roles in protein secretion and will discuss in detail the roles of mycobacterial type VII secretion chaperones in substrate recognition and targeting.

**Keywords:** chaperone; substrate recognition; protein targeting; protein secretion; type VII secretion; mycobacterium

## INTRODUCTION

Chaperones are an important group of proteins that play key roles in cellular homeostasis by assisting in protein folding, multimeric protein assembly, protein trafficking and protein degradation. In prokaryotes, three highly conserved generic chaperones, i.e. DnaK, GroEL and trigger factor (TF), are mainly responsible for preventing misfolding, premature folding and non-native interactions of cytoplasmic proteins upon their synthesis in the highly crowded environment of the cytosol (Sala, Bordes and Genevaux 2014). TF, whose most dominant substrates have been shown to be beta-barrel outer membrane proteins in *Escherichia coli* (Oh *et al.* 2011), assists the folding of newly synthesized polypeptides by preferentially interacting with short omnipresent motifs enriched in aromatic and basic residues (Patzelt *et al.* 2001). Both DnaK and GroEL are ATP-dependent chaperones that recognize short extended hydrophobic sequences, exposed during *de novo* protein folding, during stress and during protein translocation across membranes (Rudiger *et al.* 1997; Houry 2001). While DnaK has been shown to be involved in the biogenesis of some proteins with extra-cytoplasmic destinations as well (Collet *et al.* 2018), additional chaperones dedicated to the route of export are required to keep these proteins in a translocation competent state, which is often a (semi-)unfolded conformation. To export proteins out of the cytosolic compartment, bacteria have evolved distinct protein secretion systems (Costa *et al.* 2015). While several are present in almost all bacteria and transport a wide range of

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protein substrates across the cytoplasmic membrane, *i.e.* the Sec and the twin-arginine translocation (Tat) system, others are only present in a more selected group of bacterial species and only secrete a limited number of proteins. These specialized secretion systems include the type I to type VI secretion (T1 to T6S) systems that are present in Gram-negative bacteria, where they are critical for bacterial pathogenesis by secreting key virulence factors. Secretion across the Gram-negative diderm cell envelope occurs either in a one-step mechanism by a translocation channel that spans both the inner and outer membrane (i.e. in T1S, T3S, T4S and T6S), or a two-step mechanism, where the Sec and Tat system mediates transport across the inner membrane, while a separate channel mediates outer membrane transport (i.e. in T2S and T5S).

Type VII secretion (T7S) systems are related specialized secretion systems present in mycobacteria. This specific group of bacteria contains highly relevant pathogens, most notable *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Mycobacteria belong to the order of Corynebacteriales, which, in turn, is part of the large phylum of Actinobacteria, also called high GC Gram-positive bacteria. A characteristic feature of this order is the presence of a unique cell envelope that contains mycolic acids, unusually long fatty acids that can contain up to 100 carbon atoms. It is now widely accepted, amongst others based on cryo-electron microscopy (EM) imaging (Hoffmann *et al.* 2008; Zuber *et al.* 2008), that the mycolic acids are the main constituents of a second (outer) membrane. This outer membrane is highly hydrophobic and serves as an efficient permeability barrier, important for the intracellular life cycle of pathogenic mycobacteria. Nevertheless, just like other bacterial pathogens, pathogenic mycobacteria also strictly rely on extracellular proteins for their virulence. It is now clear that T7S is the major export route of these extracellular proteins in mycobacteria (Groschel *et al.* 2016). On the other hand, homologous T7S gene clusters can also be found in Actinobacteria that lack mycolic acids and more distantly related systems are present in a subset of low GC Gram-positive bacteria. Pathogenic mycobacteria can have up to five homologous T7S systems, called ESX-1 to ESX-5, that share a set of conserved components, of which four are assembled into a large, 24 subunit membrane complex (Houben *et al.* 2012; Beckham *et al.* 2017; see Fig. 3). The dimensions of the ESX-5 membrane channel, as observed by negative stain EM imaging (Beckham *et al.* 2017) dictates that the complex can only span the mycobacterial inner membrane. The mechanism of T7S substrate transport across the mycobacterial outer membrane therefore remains unknown.

An intriguing feature of T7S in mycobacteria is that the five ESX secretion systems that can be present in a single mycobacterial species each secrete their own subset of substrates that belong to several protein families. This raises the question how these related substrates are specifically recognized and targeted to the cognate secretion machinery. In recent years, it has become clear that a set of novel dedicated chaperones play crucial roles in the secretion of a specific subset of substrates via the different ESX systems (Daleke *et al.* 2012; Ekiert and Cox 2014; Korotkova *et al.* 2014). Not only are these chaperones probably involved in preventing substrate aggregation, we recently showed that they are furthermore involved in determining system specificity (Phan *et al.* 2017).

In this review, we will provide an overview of generic and specific bacterial chaperones, focusing on their mode of substrate recognition and their roles in substrate targeting to the various export machineries. Subsequently, the (potential) roles of chaperones in the recognition and targeting of the different T7S substrate families in mycobacteria will be discussed in detail.

## GENERIC SECRETION CHAPERONES

Most secretory proteins are exported either in an unfolded state via the Sec pathway or in a folded state via the Tat pathway, both mediating transport across the cytoplasmic membrane. Both Sec and Tat substrates possess and N-terminal, mildly hydrophobic, signal sequence that is cleaved upon membrane transport. Tat substrates are distinguished from Sec substrates by the presence of a conserved twin-arginine motif within their signal sequence, which mediates post-translational targeting to the Tat translocon (Palmer and Berks 2012). Many Tat substrates contain a cofactor in their mature structure, which is incorporated during the folding process in the cytosol. Folding and assembly of these Tat substrates are assisted both by the three generic molecular chaperones DnaK, GroEL and TF, and by substrate specific cytosolic chaperones, so called redox enzyme maturation proteins (REMPs) (Chan *et al.* 2015). REMPs are additionally involved in the subsequent targeting of Tat substrates to the Tat translocon (Chan *et al.* 2015).

All three generic chaperones are also involved in preventing folding of the secretory proteins that are exported via the Sec pathway (Sala, Bordes and Genevaux 2014). However, most Proteobacteria possess an additional generic chaperone, called SecB, that interacts with the Sec machinery to facilitate protein export (Sala, Bordes and Genevaux 2014). SecB is a homotetrameric chaperone that binds co- and/or post-translationally to newly synthesized proteins, maintaining them in a translocation competent state for transfer through the narrow Sec translocon (Chatzi *et al.* 2013). Crystal structures of tetrameric SecB reveals multiple binding grooves each potentially allowing the binding of ~20 amino acids-long extended polypeptides (Sala, Bordes and Genevaux 2014). SecB does not specifically recognize signal sequences (Gannon, Li and Kumamoto 1989) and portions of the mature part of substrates are probably wrapped around the chaperone tetramer (Khisty, Munske and Randall 1995; Crane *et al.* 2006). It is considered a promiscuous chaperone, recognizing short sequences enriched in aromatic and basic residues (Knoblauch *et al.* 1999), and is therefore postulated to be involved in folding of cytosolic proteins as well (Ullers *et al.* 2004). SecB is involved in targeting of secretory proteins to the Sec translocon via its specific interaction with SecA, the ATPase and motor protein of the system (Hartl *et al.* 1990). The observation that the interaction sites of SecB with the substrate and with SecA significantly overlap hints towards a mechanism of substrate transfer from SecB to the Sec translocon (Crane *et al.* 2005).

In addition to the transport of secretory proteins, the Sec translocon is, together with the insertase YidC, involved in membrane insertion of inner membrane proteins (Luirink *et al.* 2012). As membrane proteins have the high tendency to aggregate due to their hydrophobic nature, they are targeted in a co-translation fashion by the ribosome associated and highly conserved Signal Recognition Particle (SRP), consisting of both protein and RNA (Akopian *et al.* 2013). SRP binds to sufficiently hydrophobic sequences as soon as they emerge from the ribosome and targets the ribosome-nascent chain complexes to the Sec translocon, through the interaction with its membrane receptor, the GTPase FtsY.

## SPECIFIC SECRETION CHAPERONES

In contrast to the generic chaperones that are able to bind a wide range of substrates, highly specific chaperones usually interact with substrates that are secreted by the specialized secretion systems. The best-described examples of these specific chaperones are those found in T3S that mediate the direct injection of proteins, referred to as the effector proteins, into host cells. The translocation of T3S substrates across both the bacterial cell envelope and the host membrane is mediated in a one-step mechanism using large, needle-like nanomachines (Deng *et al.* 2017).

T3S mediates the export of three distinctive protein groups in a highly sequential manner: first the needle subunits (early substrates) are exported, followed by the pore-forming subunits that puncture the host membrane (intermediate substrates), after which the actual effector proteins (late substrates) are translocated. Each substrate group depends on specific chaperones for successful export. Needle subunits interact with a structurally conserved chaperone pair to prevent premature self-assembly through their amphipathic C-terminal helix (Quinaud *et al.* 2007). Also the hydrophobic pore-forming substrates strictly depend on conserved homodimeric chaperones, classified as Class II chaperones, to prevent their premature assembly and degradation (Menard, Sansonetti and Parsot 1994). The Class II chaperone-substrate interface is conserved and consists of a scaffold containing tetratricopeptide repeat (TPR) motifs, known for their involvement in protein–protein interactions, and an N-terminal hydrophilic chaperone binding domain (CBD) (Lunelli *et al.* 2009). However, most information on the mode of substrate binding and, in particular, the role of T3S chaperones in substrate targeting to the transport machinery has been obtained for the effector chaperones, referred to as the Class I chaperones. The majority of these chaperones (the Class IA chaperones) are highly specific, serving a single substrate. They are small, usually dimeric, and share very low sequence identity of ∼20%, but show striking structural similarities.

T3S effector substrates typically contain non-cleavable, 15–20 residue long secretion signals at their N termini, although no clear consensus sequence for these regions has yet been identified (Wilharm *et al.* 2007). C-terminal from this secretion signal lies the CBD that encompasses 50–100 amino acids. While the N-terminal secretion signal is sufficient for protein secretion (Lloyd *et al.* 2001), the CBD directs substrates to the cognate needle complex (Cheng, Anderson and Schneewind 1997; Lee and Galan 2004). T3S chaperones probably interact with this CBD via extended hydrophobic surface areas (He, Nomura and Whittam 2004). Crystallography analysis of different chaperone-substrate complexes shows that not only the fold of chaperones, but also the binding of the CBDs, wrapped around the chaperone dimer, follows a conserved principle (Stebbins and Galan 2001; Birtalan, Phillips and Ghosh 2002; Phan, Austin and Waugh 2005). The functional significance of the conserved chaperone-CBD structure is not clear. The chaperone SycO of *Yersinia* has been shown to keep its substrate YopO in a translocation competent state, as it is prone to aggregate, due to its hydrophobic domain that is essential for its proper localization after translocation into host cells (Letzelter *et al.* 2006). However, nuclear magnetic resonance analysis of the structural and dynamic changes in the *Yersinia* effector YopE upon binding of its chaperone SycE revealed that rather than maintaining an unfolded state in the effector, the secretion chaperone promotes structuring of the CBD (Rodgers *et al.* 2010). This supports the hypothesis that the CBD together with the chaperone constitutes a three-dimensional targeting signal (Birtalan, Phillips and Ghosh 2002).

## MYCOBACTERIAL T7S CHAPERONES

### T7S substrates

All T7S substrates secreted by the five homologous T7S systems in mycobacteria can be divided into three protein families, the Esx, the PE/PPE and the Esp proteins. Esx genes are conserved in a wide range of bacteria and are also substrates of the T7S-like systems in low GC Gram-positive bacteria. In contrast, *pe/ppe* genes are mainly found in mycolic acid containing bacteria and Esp proteins are specific for mycobacteria (Houben, Korotkov and Bitter 2014). Interestingly, in mycobacteria different secretion systems are able to transport members of the Esx and PE/PPE proteins, while Esp proteins are specifically associated with the ESX-1 system. The Esx substrates all have a size of ∼100 amino acids, whereas the Esp and especially the PE/PPE proteins greatly vary in length. All Esx proteins and some PE/PPE and Esp substrates are translated from a bicistronic transcript (Gey Van Pittius *et al.* 2001; Gey van Pittius *et al.* 2006). These co-transcribed substrates form heterodimers in the cytosol and are thought to be secreted as (partially) folded heterodimers, as they are co-dependent on each other for secretion. Examples of heterodimeric substrates are the ESX-1 dependent heterodimers EsxA/EsxB (Renshaw *et al.* 2005) and EspA/EspC (Lou *et al.* 2017), and the ESX-5 dependent PE25/PPE41 (Strong *et al.* 2006). Although belonging to different protein families both Esx and PE/PPE pairs show a striking structural resemblance (Fig. 1; Houben, Korotkov and Bitter 2014). Solved structures of different Esx pairs show a highly conserved fold, in which dimerization is mediated by a helix-turn-helix motif of both proteins, oriented in an antiparallel manner (Fig. 1; Renshaw *et al.* 2005; Arbing *et al.* 2010; Ilghari *et al.* 2011). The double helix structures cover almost the complete protein sequences, excluding short flexible N- and C-termini. This is different for the PE and PPE proteins, which are named referring to the presence of a conserved

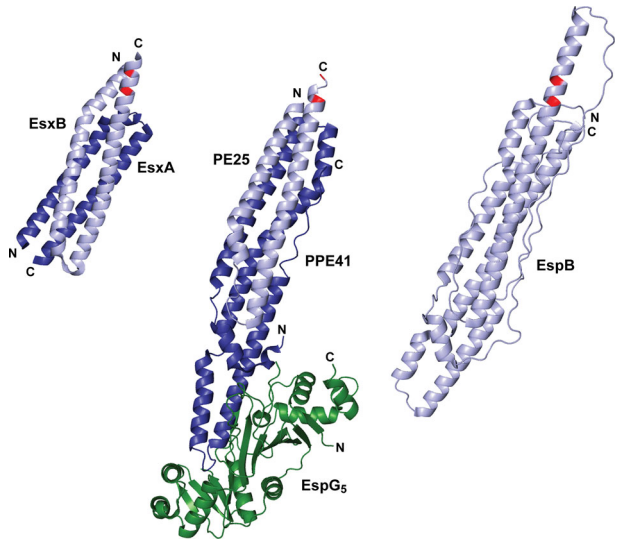


**Figure 1.** Crystal structures of representative T7S substrates showing conserved folds and the EspG binding site. Ribbon representations of EsxA/EsxB of *M. tuberculosis* (PDB 3FAV; Poulsen *et al.* 2014), PE25-PPE41 in complex with their chaperone $EspG_5$ of *M. tuberculosis* (PDB 4KXR; Korotkova *et al.* 2014), and EspB from *Mycobacterium smegmatis* (4WJ1; Solomonson *et al.* 2015). Notably, the C-terminal 11, 12, 8, and 20 residues of EsxA, EsxB, PE25, and PPE41, respectively, and the N-terminal 6 amino acids of PE25 are disordered in the structures. The structure of EspB lacks a C-terminal domain of 233 residues. Secretion signal motifs YxxxD/E (Daleke *et al.* 2012) are shown in red. N, N-terminus; C, C-terminus.

proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motif. For these proteins, only the N-terminal ~110 and ~180 amino acids long PE and PPE domains, respectively, are highly conserved on sequence and structural level (Fig. 1; Strong *et al.* 2006; Chen *et al.* 2017). For the more variable C-terminal domains (Gey van Pittius *et al.* 2006) no structural information is currently available. While the PE domain consists of two alpha helices, the PPE domain contains five alpha-helices, of which helix $\alpha$2 and $\alpha$3 mediate dimerization with the PE partner, forming the four-helix bundle similar to Esx pairs (Strong *et al.* 2006; Chen *et al.* 2017). The fourth and fifth alpha helix of the PPE domain form an extending hydrophobic tip. Of the final group of substrates, the ESX-1 specific Esp proteins, only structural information is available for monomeric EspB (Fig. 1; Korotkova *et al.* 2014; Solomonson *et al.* 2015). Interestingly, the EspB structure shows a PE/PPE-dimer like fold, suggesting this protein is secreted as a monomer. In addition, structural predictions by Phyre[2] (Kelley *et al.* 2015) and SWISS-MODEL of dimeric Esp substrates, such as EspA/EspC and EspE/EspF, indicate similar alpha-helical folds as PE/PPE proteins, suggesting this is a conserved structural feature of all T7S substrate families.

The Esx, PE and Esp substrates all have a conserved secretion motif, containing a tyrosine residue and a negatively charged residue separated by 3 random amino acids (YxxxD/E), which is essential for secretion. This motif is positioned directly C-terminal to the helix-turn-helix of one of the partner proteins, for example, in EsxB for EsxA/EsxB, EspC for EspA/EspC and always in the PE proteins for PE/PPE dimers (Fig. 1; Daleke *et al.* 2012). The secretion signal is possibly longer than the YxxxD/E motif, consisting of additional hydrophobic residues that together with the conserved Y and D/E residues are aligned at one side of the C-terminal part of alpha helix 2 (Poulsen *et al.* 2014). Accordingly, the most C-terminal amino acids of EsxB, C-terminal to the YxxxD/E motif, are required for secretion (Champion *et al.* 2006) and are able to interact with the membrane complex associated ATPase EccC, involved in substrate recognition and transport (Rosenberg *et al.* 2015; see Fig. 3). Swapping the C-terminal region containing the secretion signal of two PE substrates of different ESX systems does not affect their secretion nor system specificity in the model organism *Mycobacterium marinum*, suggesting this motif is a general secretion signal in T7S (Daleke *et al.* 2012).

## Possible chaperone roles of specific substrates

While it is still unclear to what extent heterodimeric substrates remain intact after secretion is completed, it is possible that one of the proteins, for example, the protein containing the general secretion motif, acts as the chaperone to facilitate the secretion of the partner protein. Indeed, whereas for EsxA a distinctive role in virulence has been described, this is not the case for EsxB, the protein that contains the general secretion motif (de Jonge *et al.* 2007; Smith *et al.* 2008). EsxA and EsxB have an extensive hydrophobic contact surface and form a tight ($K_d \leq 11$ nM) soluble complex when co-expressed (Renshaw *et al.* 2002; Renshaw *et al.* 2005). Interestingly, while monomeric EsxB is soluble and relatively unstructured when expressed in *Escherichia coli* in the absence of EsxA, monomeric EsxA is highly insoluble when expressed without EsxB (Renshaw *et al.* 2002), supporting the hypothesis that EsxB prevents aggregation of EsxA. The unusual feature of these potential chaperones that they are co-secreted with their specific substrates could be explained by the hypothesis that they are additionally required to translocate the substrates across the mycobacterial outer membrane.

Another intriguing feature of T7S substrates, particularly of the ESX-1 system, is that not only paired but also unpaired substrates are co-dependent on each other for secretion, for example, the secretion of EspA/EspC in *M. tuberculosis* is affected by mutations in *esxA/esxB* and *vice versa* (Fortune *et al.* 2005; Champion *et al.* 2009). Although most extensively documented for ESX-1, also some level of co-dependency has been observed for other ESX systems (Shah and Briken 2016; Tufariello *et al.* 2016; Ates *et al.* 2018). In particular, a four-gene region, duplicated from the *esx-5* gene locus and encoding two Esx, a PE and a PPE protein, but also the ESX-5 substrate PPE38 have been shown to be crucial for the ESX-5 dependent secretion of the major subgroup of PE substrates, the so-called PE_PGRS proteins (Shah and Briken 2016; Ates *et al.* 2018). While the extend of this co-dependency is still not clear, the general notion emerges that the Esx substrates are strictly required for the secretion of the other substrates of the same system. Together with the fact that the Esx proteins are amongst the most conserved T7S proteins, this raises the possibility that Esx pairs are involved in facilitating the secretion of the other substrate families, perhaps by a chaperone-like activity. As there is still no clear insight into the mechanism of and hierarchy in substrate co-dependency, it complicates not only the analysis of the role of individual substrates, but also the mechanism of T7S, as a mutation in, for example, specific chaperones will also affect the secretion of substrates that do not interact with this chaperone.

## EspG, a dedicated chaperone for PE/PPE substrates

Unlike Esx pairs that are usually soluble when co-expressed in *E. coli*, most PE/PPE pairs are insoluble when co-expressed under similar conditions, also when their variably C-terminal domains are deleted (Strong *et al.* 2006). Evidence is accumulating that a dedicated chaperone, called EspG, is keeping members of this substrate family in a translocation competent state. Notably, the name Esp (ESX-1 specific protein) was given to all proteins, secreted and non-secreted, that were thought to be specific for the ESX-1 system (Bitter *et al.* 2009). While EspG was initially also considered an Esp, we now know that four of the five ESX systems contain an EspG protein, albeit with very low amino acid conservation. As also seen for, for example, T3S effector chaperones, these EspG proteins are despite this low conservation highly similar in structure (Fig. 2; Ekiert and Cox 2014; Korotkova *et al.* 2014). While initial studies already illustrated the importance of $EspG_5$ in the secretion of several ESX-5 substrates in the model organism *M. marinum* (Abdallah *et al.* 2009), the observation that the deletion of $espG_1$ decreases the stability of the ESX-1 substrate PPE68 in *M. tuberculosis* (Bottai *et al.* 2011) was the first hint of a specific chaperone-like activity. Subsequently, EspG chaperones were shown to specifically interact with PE/PPE pairs that are secreted by the respective secretion system in *M. marinum* (Daleke *et al.* 2012). In addition, co-expression of EspG-PE/PPE sets in *E. coli* were shown to increase the solubility of the cognate PE/PPE substrates (Korotkova *et al.* 2014). Based on these data, together with the observation that EspG is strictly cytoplasmic, it was hypothesized that EspG is required for the recruitment of the PE/PPE pairs to the cognate membrane embedded secretion complex, after which it dissociates from these substrates (Daleke *et al.* 2012). The crystal structure of $EspG_5$ in complex with the PE25/PPE41 dimer subsequently revealed that the chaperone exclusively binds to the hydrophobic tip of helices $\alpha$4 and $\alpha$5 of the PPE domain of PPE41 (Fig. 1; Ekiert and Cox 2014; Korotkova *et al.* 2014). The observation that binding of $EspG_5$ does not introduce conformational changes to the PE/PPE

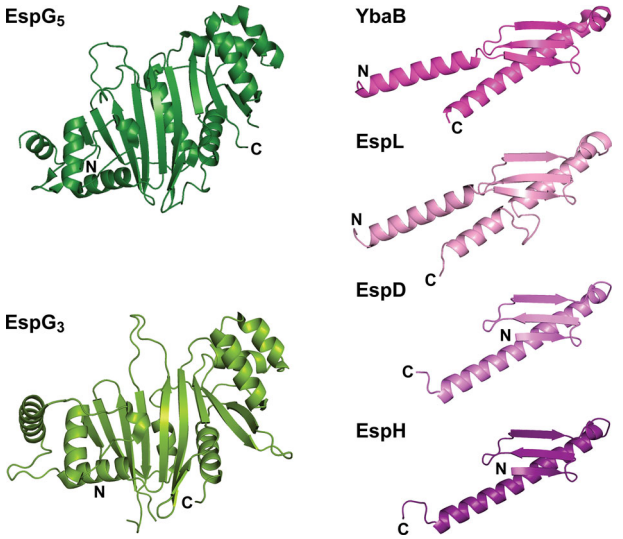


**Figure 2.** Structures of (potential) T7S chaperones. Ribbon representations of the solved crystal structures of $EspG_5$ of *M. tuberculosis* (selected from PDB 4KXR; Korotkova *et al.* 2014), $EspG_3$ of *M. tuberculosis* (4W4I; Ekiert and Cox 2014), and YbaB from *H. influenzae* (PDB 1J8B; Lim *et al.* 2003), and predicted homologous models to YbaB of EspL (coverage of by 81% by residue 10-109; produced by SWISS-MODEL; Tian *et al.* 2016), EspD (coverage of 38% by residue 65-134; produced by Phyre2) and EspH (coverage of 39% by residue 66-137; produced by Phyre2) of *M. tuberculosis*. Notably, although YbaB and EspL are able to form homodimers (Lim *et al.* 2003; Tian *et al.* 2016), this has not been observed for EspH (Phan, van Leeuwen *et al.*, submitted). We therefore only show the monomeric structures for these proteins to emphasize the structural resemblance. N, N-terminus; C, C-terminus.

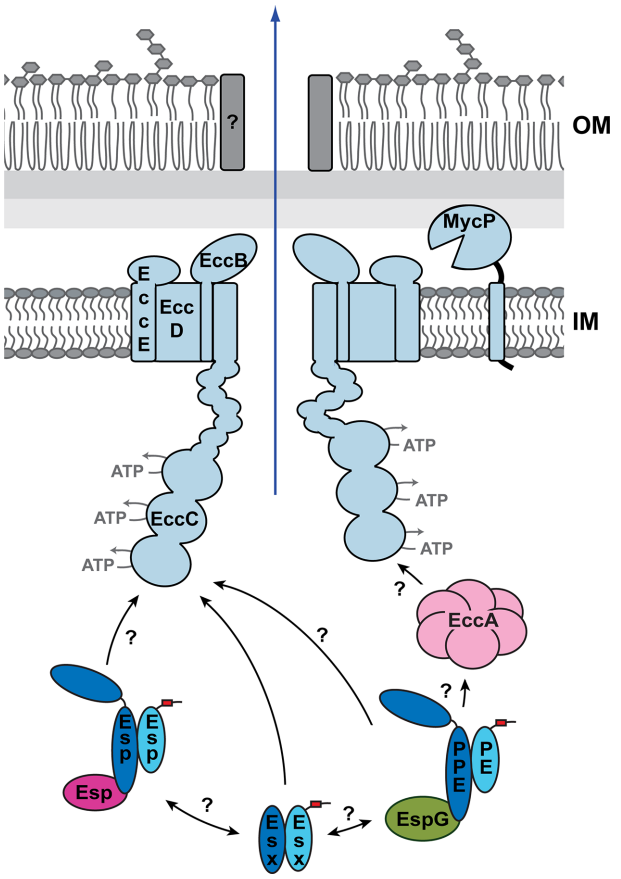


**Figure 3.** Working model for substrate recognition and targeting in type VII secretion. The three T7S substrate families, the Esp, Esx, and PE/PPE proteins, are generally exported as heterodimers by the T7S secretion machinery. While dimerization occurs via a conserved four-helix bundle fold, Esp, and PE/PPE proteins additionally contain highly variable C-terminal domains. Substrate recognition occurs via a C-terminal secretion motif on one of the dimer subunits (indicated by a red box). The cytosolic component EspG specifically recognizes a conserved hydrophobic helical tip in PPE proteins and possibly mediates targeting of PE/PPE substrates to the cognate membrane channel. Esp substrates also require binding of dedicated chaperones, possibly to a similar helical tip structure, to prevent their premature self-assembly. While the core membrane channel consists of the conserved membrane components EccB, EccC, EccD, and EccE (Houben *et al.* 2012; Beckham *et al.* 2017), the fifth conserved membrane component, the protease MycP, while essential for secretion, is not an integral part of this complex (van Winden *et al.* 2016). The three nucleotide binding domains of EccC are likely involved in energizing translocation of substrates through this channel. In this model, the T7S membrane complex mediates transport only across the inner membrane, while a so far unidentified separate channel mediates translocation across the outer membrane. The observed interaction between the secretion signal of an Esx pair with EccC *in vitro* (Champion *et al.* 2006; Rosenberg *et al.* 2015) is indicated by an arrow. Arrows with question marks indicate the potential interactions between the different substrate families, of the other substrates with EccC and of the postulated specific interaction of PE/PPE pairs with the cytosolic ATPase EccA. IM, inner membrane; OM, mycobacterial outer membrane.

dimer, but increases the solubility of the protein pair (Korotkova *et al.* 2014), suggests that EspG prevents self-aggregation via the hydrophobic tip of the PPE protein. This potential function of EspG chaperones is similar to that of the T3S effector chaperone SycE that prevents aggregation of its substrate YopO (Letzelter *et al.* 2006).

Based on the observation that EspG proteins specifically interact with PE/PPE pairs of their respective system, EspG was hypothesized to be involved in determining system specificity of these substrates. Indeed, we recently showed that the ESX-1 dependent substrate pair PE35/PPE68_1 could be rerouted to the ESX-5 system of *M. marinum* by replacing the $EspG_1$ binding domain of PPE68_1 with the equivalent $EspG_5$ binding domain of the ESX-5 substrate PPE18 (Phan *et al.* 2017). This domain replacement makes the PE35/PPE68_1 protein pair independent of both $EspG_1$ and the ESX-1 membrane complex but instead dependent on $EspG_5$ and the ESX-5 complex for secretion. These findings indicate that EspG not only is required for the solubility of PE/PPE complexes, but also that it specifically directs these protein pairs to their respective ESX-system. A similar role in determining system specificity has been observed for the CBD of T3S effectors (Lee and Galan 2004). The question remains how the other substrate groups, especially the Esx proteins that lack a hydrophobic helical tip, are specifically recognized. While their C-terminal secretion signal could still be involved, another possibility is that Esx proteins are guided to the cognate secretion machinery by other substrates of the same system (see Fig. 3).

## EccA

The second ESX conserved cytosolic component, which could potentially execute a chaperone-like activity, is EccA, an ATPase belonging to the AAA+ (ATPases Associated with diverse cellular Activities) protein family. All EccA homologs consist of two domains with the C-terminal domain containing the typical AAA+ ATPase characteristics, such as the walker and oligomerization motifs, and the N-terminal domain containing 6 tandem TPR motifs (Wagner, Evans and Korotkov 2013). Similar to other AAA+ ATPases, the ATPase domain of $EccA_1$ has been shown to mediate homohexamerization (Luthra *et al.* 2008). The role of EccA in secretion remains unclear. While some studies showed that $EccA_1$ and $EccA_5$ are required for the secretion of different

ESX-1 and ESX-5 substrates, respectively (Gao *et al.* 2004; Abdallah *et al.* 2006; Bottai *et al.* 2012; Joshi *et al.* 2012), other studies showed that they are dispensable for secretion (Converse and Cox 2005; Houben *et al.* 2012). This discrepancy could be explained by our recent observation that the importance of $EccA_1$ for ESX-1 mediated secretion in *M. marinum* varies between different growth media (Phan, van Leeuwen *et al.*, in press). In line with the central role of an AAA+ ATPase in the disassembly the T6S secretion apparatus (Bonemann *et al.* 2009), a role of EccA in the disassembly of the EspG-PE/PPE complex upon targeting to the T7S membrane complex has been proposed (Ekiert and Cox 2014). This hypothesis is supported by the observation that EccA proteins are encoded only in ESX gene clusters that also encode PE and PPE proteins (Houben, Korotkov and Bitter 2014).

### Specific chaperones for Esp substrates

As already mentioned, the structure of monomeric EspB shows striking structural resemblance to PE/PPE pairs, including the presence of a two-helical hydrophobic tip (Fig. 1; Korotkova *et al.* 2015; Solomonson *et al.* 2015). Interestingly, EspB has been shown to multimerize upon secretion and forms heptameric ring-shaped particles upon overexpression in *E. coli*, as visualized by negative stain EM (Korotkova *et al.* 2015; Solomonson *et al.* 2015). Modeling the solved crystal structure of monomeric EspB within these EM images produces an arrangement where the hydrophobic helical tips of EspB are tightly packed. From this finding, it was postulated that a specific chaperone is required to prevent premature self-assembly via this hydrophobic tip, similarly as EspG for PE/PPE substrates. In this respect, the putative chaperone for EspB could have a comparable function as the chaperones of T3S pore-forming substrates (see above).

While a dedicated chaperone for EspB has not been identified yet, stable expression of the substrate pair EspA/EspC in *M. tuberculosis* is dependent on the presence of another ESX-1 associated protein EspD, that is co-transcribed with the substrate pair (Chen *et al.* 2012). This indicates that EspD is a dedicated chaperone for EspA/EspC. EspD shows 55% sequence identity with another small protein, EspH, encoded from a gene sharing an operon structure with $espG_1$ and $eccA_1$. Our recent analysis of an *espH* deletion mutant in *M. marinum* shows an abolished expression and secretion of EspE/EspF and we could furthermore show that EspH remains in the mycobacterial cytosol, where it specifically interacts with EspE (Phan, van Leeuwen *et al.*, in press). Using Phyre[2] (Kelley *et al.* 2015), we discovered that EspH and EspD are predicted to share structural similarity to YbaB of *Haemophilus influenza* and *E. coli*, a widely-distributed DNA-binding protein involved in regulation of gene expression (Fig. 2; Lim *et al.* 2003; Cooley *et al.* 2009). However, more recent studies, showing that YbaB of *E. coli* interacts and is a target of ClpYQ proteases (Tsai *et al.* 2017) and enhances heterologous membrane protein production (Skretas and Georgiou 2010), indicate a more complex function. Interestingly, a structural study of the ESX-1 associated EspL also revealed a high resemblance to YbaB (Fig. 2; Tian *et al.* 2016), making it tempting to speculate that EspL also functions as a chaperone. It is still unclear though why these Esp proteins might have a similar fold as a DNA-binding protein. While YbaB and EspL form homodimers when expressed in *E. coli*, we were unable to detect dimerization of EspH upon heterologous expression in *M. marinum* (Phan and Houben, unpublished results). It therefore remains uncertain whether these potential structurally conserved Esp chaperones function as dimers or as monomers.

## CONCLUSIONS

Chaperones, in general, interact with their substrates either to ensure their proper folding or to prevent premature folding and/or assembly to maintain their competence for translocation. However, while the generic chaperones TF, DnaK, GroEL and SecB are highly promiscuous in substrate recognition and bind a wide range of proteins, the chaperones involved in specialized secretion systems are usually more specific for a substrate subgroup or even a single protein. This difference could be linked to the observation that secretion via specialized secretion systems often occurs in a highly regulated fashion, requiring specific triggers such as target cell contact, and that different substrate classes are secreted in a more hierarchical fashion. Dedicated chaperones could play a central role in both these processes, by regulating the targeting of different substrate classes to the translocation machinery. For T7S in mycobacteria, the mechanism of substrate recognition and targeting seems to be highly complex (Fig. 3). Not only are different substrate families dependent on each other for secretion, they furthermore rely on dedicated chaperones for successful export by the cognate secretion machinery. The elucidation of the roles of the different chaperones in system specific substrate recognition and targeting will be instrumental to understand the mechanism of T7S across the specific mycobacterial cell envelope.

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