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Strapline: Structural Biology

Title: **Mycobacterial ESX secrets revealed**

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Standfirst (330 characters):

Structural analysis of the mycobacterial ESX-5 secretion complex presents an important step towards understanding how the ESX type VII (T7) secretion systems can translocate a multitude of substrates—including virulence factors involved in pathogenesis—across the bacterial cell envelope.

Main Text (800-900 words):

Mycobacterium tuberculosis, the agent of tuberculosis, is one of the top 10 causes of death worldwide, accounting for over 10 million infections in 2015[1]. The interaction of *M. tuberculosis* with its human host is modulated by a multitude of proteins that are secreted across the complex bacterial cell envelope[2], which comprises a cytoplasmic membrane and an unusual mycolic acid-containing outer membrane that is covalently linked to peptidoglycan and arabinogalactan [3] (Fig 1). Translocation across this barrier requires specialized secretion machines variously termed ESX or type VII (T7) secretion systems[4].

M. tuberculosis encodes five paralogous ESX secretion systems, with ESX-1, ESX-3 and ESX-5 being essential for full virulence[2]. Collectively, these ESX systems secrete hundreds of proteins; two of the biggest families of ESX substrates are the Pro-Glu (PE) and Pro-Pro-Glu (PPE) proteins that are particularly abundant in pathogenic mycobacteria and many of these are secreted though ESX-5. ESX systems comprise five essential membrane components – EccB, EccC, EccD, EccE which form a core complex[5] and a peripherally-associated serine protease mycosin, MycP[6]. EccC is the only component of the core complex with known function. It is a membrane-bound ATPase of the FtsK/SpoIIIE family with a rigid array of three P-loop ATPases that protrude into the cytoplasm, the most C-terminal of which interacts with the signal sequence of the EsxB substrate[8]. EsxB docking has been reported to promote hexamerization of EccC, suggesting that assembly of the machinery may be regulated by substrate binding. The stability and overall structure of core complex, however, remains unclear.

In this issue of *Nature Microbiology*, Beckham *et al*.[7] use single particle negative stain electron microscopy to reveal the first structural information of the cytoplasmic membrane complex of mycobacterial ESX-5. They produced and isolated recombinant *Mycobacterium xenopi* ESX-5 complexes comprising EccB, EccC, EccD and EccE from *M. smegmatis,* This 1.5MDa complex was stable in the absence of cognate secretion substrates indicating that contrary to studies with isolated EccC, assembly of the core complex was substrate-independent. Single particle negative-stain electron microscopy analysis followed by 3D reconstruction revealed a complex with 6-fold symmetry, in keeping with the hexameric arrangement of EccC observed previously[8]. The bulk of the complex forms a dense, compact structure, but long projections could be seen emanating from individual particles. Immunogold labelling of single particles confirmed that these projections corresponded to the cytosolic ATPase domains of EccC, helping to place EccC at the centre of the hexameric arrangement (Fig 1). This is consistent with EccC forming the central pore, with the ATPase domains forming concentric stacking hexameric rings that may act as a tube to funnel substrates into the membrane channel[8,9].

A prior X-ray crystal structure of the membrane-extrinsic domain of EccB[10] could be docked into density at the periplasmic side of the complex, suggesting that this protein forms an extra-cytoplasmic collar around the pore. This could potentially seal the channel at the periplasmic exit (Fig 1). Further periplasmic density was contributed by EccE which was shown to be located at the periphery of the complex. This careful mapping of individual components within the structure allowed Beckham *et al*.[5] to propose an overall organisation for the secretion machinery where the EccC-EccB-EccD-EccE proteins essentially form four concentric hexameric rings. The structure provides a framework for the design of experiments to assess the roles of individual components within the complex, and to propose models to describe how protein secretion is achieved.

One such model is shown in Fig 1, where EccC is proposed to exist as a homo-hexamer through interactions within the transmembrane domains, while the cytoplasmic ATPase domains of each EccC subunit reside as monomers. This satisfies earlier studies showing that the membrane-extrinsic region of EccC is monomeric when crystallised[8] and is in agreement with the findings of Beckham *et al*.[5] that this region of EccC can adopt multiple conformations in single particles, suggesting this region is highly flexible in the resting state. Upon substrate binding the ATPase domains of EccC could then oligomerize[8], and modelling a multimer of the ATPase domains of EssC (from the distantly-related T7 secretion system of Gram-positive firmicutes) suggests that this would form a channel inside the hexamer of approximately 30 Å[9]. The vast majority of T7 secretion substrates appear to comprise at least one domain that can form a helical hairpin structure[11] with folded dimensions that can be accommodated in the channel, even when present as a potential interlocked dimer[12] (Fig 1). The precise role for ATP binding and hydrolysis and how this is transduced to the mechanical movement of proteins across the membrane is currently unknown and is an area that is ripe for future research.

The structure of the ESX-5 membrane complex presented by Beckham *et al*.[5] also raises some intriguing questions. For example, the complex dimensions suggest it may only span the cytoplasmic membrane of mycobacteria, yet substrate proteins must also cross the outer mycolic-acid layer. It now seems highly unlikely that any of the EccB, EccC, EccD and EccE components are involved in this process, so how is this achieved? Secondly, mycosin proteases are membrane-bound components of ESX secretion systems that are essential for secretion and are also implicated in stabilizing the membrane complex[7] – how do they associate with the membrane complex and what is their role in protein secretion? Finally, a related T7 secretion system in firmicutes shares a common EccC/EssC membrane-bound ATPase component with the ESX system, but otherwise lacks clear homologues of any of the other mycobacterial membrane components[13]. It will be interesting to see whether these distantly related secretion machineries share a common architecture and mode of action.

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Figure legend:

Figure 1. Speculative model for ESX-5 organisation under resting and translocating conditions. In this model, EccC forms the translocation channel, consistent with the conservation of this ATPase across all Type VII secretion systems and its localization near the centre of the complex in the single particle EM reconstruction of ESX-5. In the resting state, the ATPase domains of EccC are in a monomeric arrangement, and the channel is

sealed at the periplasmic side by EccB. Upon interaction with the C-terminal signal sequence of a heterodimeric substrate protein, EccC hexamerises and the substrate is translocated across the cytoplasmic membrane in a folded state. The three P-loop ATPase domains are each shown with a generic bound nucleotide (black circle) as the precise role of nucleotide binding and hydrolysis for each domain during substrate binding and translocation is currently unclear.The role of the MycP protease and how substrates cross the mycolic-acid containing outer layer also remain unknown.

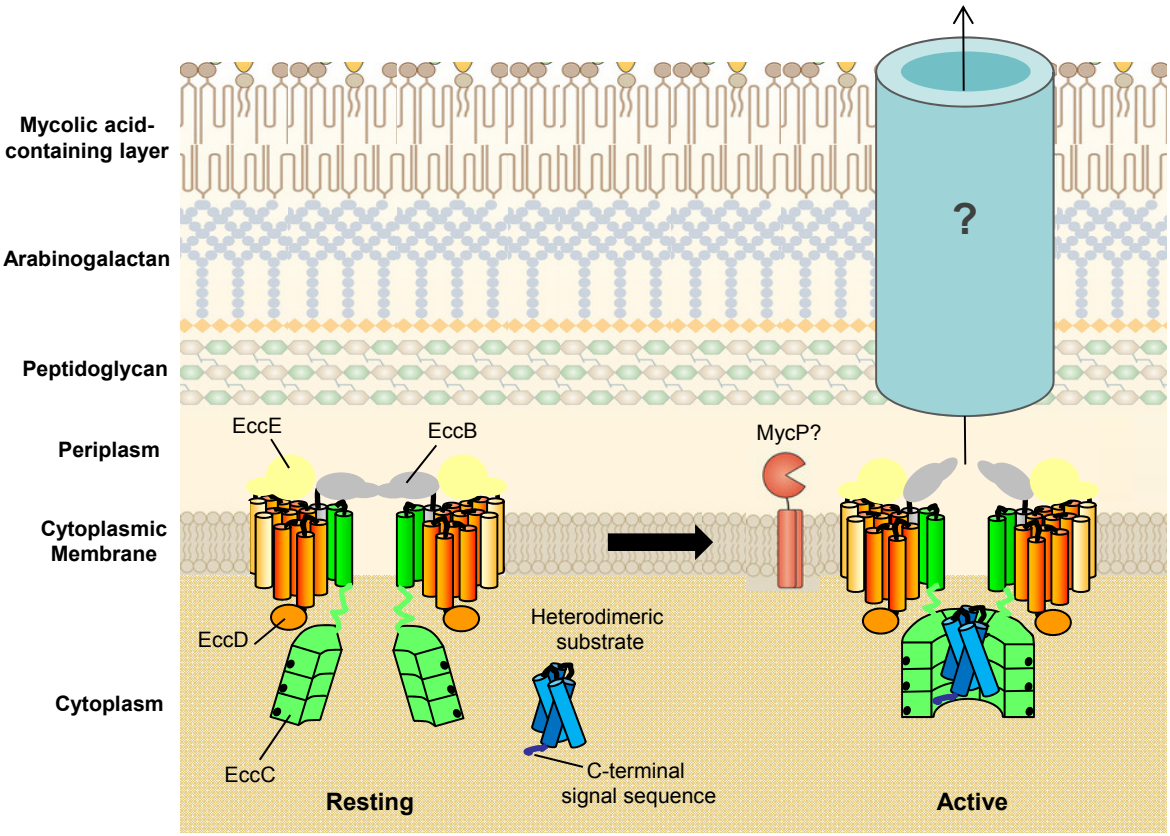
Mycolic acid-containing layer
Arabinogalactan
Peptidoglycan
Periplasm
Cytoplasmic Membrane
Cytoplasm
EccE
EccB
EccD
EccC
MycP?
Heterodimeric substrate
C-terminal signal sequence
?
Resting
Active

Figure 1