

Deciphering the role of mycobacterial secreted proteins through structural biology

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Abril Gijsbers

I. Economical and societal impact

TB is the oldest disease on record, and still, too many patients succumb from it every year due to ineffective treatment. The development of new drugs has been challenging due to the complex infection process, the pathogen protecting itself in the interior of the host cells, and the variability of disease severity between patients linked to the different lineages (290). Nevertheless, the world would have a great benefit from an efficient therapy to prevent and treat TB. 1) Annual investment (US\$ 5 billion) in health care and medical units dedicated to TB, including personnel during and after hospitalisation, can be used to fulfil other medical or social needs. 2) TB fractures fragile economies by disabling a considerable number of people to work, compromising small business and family financials. Reducing patients with active infection could aid the country's growth by contributing to the workforce and saving families from crossing the impoverishment threshold. 3) The latter has more profound consequences; life quality would profit from poverty reduction, which could mitigate the stigma around the disease helping in social re-integration. Only a few pharmaceutical companies have shown interest in engaging this challenge (291). The reason is the little revenue expected from treating a disease with an available treatment combined with a market dominated by middle and low-income countries, leaving the task in the hands of academia with relative plodding progress. However, it is of most importance to have a joined front, like the one seen for COVID-19, if we want to win this long war against TB. Otherwise, MDR and XDR-TB threat to turn **this silent pandemic into a thunderous one**.

The new vaccines that target the Spike protein of the SARS-CoV2 are a few examples of how structural biology has changed how new therapeutics are developed, speeding the process by making a structure-based drug and vaccine design (292,293). However, this has been possible after decades of hardware and software development. Nevertheless, every protein represents a new challenge, limiting our capacity to determine protein structures. New developments or tools, like the VitroJet with a robust standard protein (BfrB) to test it, will potentially take the field of structural biology, in particularly cryo-EM, to characterise any protein or protein complex, and give a solution to many medical or biological problems, improving people's lives. In this thesis, I described the structural characterisation of three mycobacterial proteins necessary for the pathogenesis of *M. tuberculosis*, which could be target for treating TB. BfrB is a ferritin protein that works as a vault, storing iron for vital cellular processes. During infection, the host cell limits the concentration of iron to make the bacterium "starve". Disruption of BfrB function would compromise the survival of *M. tuberculosis* and the progression of the infection in this hostile environment, as preliminary data has shown (294). On the other hand, EspB and EspK belong to the ESX-1 secretion system, a protein complex responsible for the translocation of *M. tuberculosis* from the phagosome to the host cell's cytoplasm (74). This event is a characteristic of all pathogenic species and does not depend on genomic variations (lineages). Likewise, mutations in *espB* and *espK* genes are known to result in the attenuation of the pathogen. As secreted proteins

accessible to the environment, they could be good targets for structure-based drug and vaccine design.

2. Scientific impact

Mycobacterium tuberculosis has walked through history with us, killing more humans than any other pathogen. Despite the arduous work to understand the different mechanisms that *M. tuberculosis* puts in action to evade the immune system, we are far from succeeding. The subjects of this thesis are mycobacterial proteins that play a role in the survival of the bacteria during infection, particularly proteins belonging to the T7SS. Which role and how the proteins perform it were the questions that drove this research. The involvement of the T7SSs in different steps of pathogenesis has made them considered one of the most important virulence factors, attracting many scientists and making this a very competitive field. However, this has not been enough to speed the pace of its characterisation. The T7SS or the ESX-systems are protein complexes embedded in the membrane of mycobacteria, which has a double complication. 1) Lipids composition could be critical for the proper structure of the complex. Mycobacteria have an unusual membrane composition (295), which might explain why the successful purification of the machinery has only been reported from mycobacterial species. Nevertheless, that is not a simple answer to our problems. Mycobacteria is not a common organism for recombinant protein expression; hence there are limited molecular-biology tools compared to other model organisms and expertise. That is why recombinant work in heterologous systems like *E. coli* is still a common practice. 2) The ESX-1 system has six proteins that form the inner-membrane, 17 proteins that are secreted or regulate secretion, and other multiple proteins that regulate the system at the expression level. To understand how the machinery works, we need to identify all the elements involved and understand their function, which might implicate working in settings with multiple proteins simultaneously. Moreover, to put another level of difficulty, as expressed before, TB is caused by different mycobacterial lineages that have polymorphisms across their genome, changing the behaviour of proteins.

The elucidation of ESX-3 (166,167) and ESX-5 (170,171) structures are milestones for the scientific community, giving valuable insight into the secretion mechanism for all five systems (296). Unfortunately, they are not enough to understand the whole process, for example, because they cannot explain how proteins will be secreted through the outer membrane. The proteins that compose the outer-membrane complex are unknown, information critical for the proper understanding of the system; secreted proteins have to traverse the physical barrier, and ESX-1 cytolytic activity is carried out through physical contact (176). The subject's relevance highlights the importance of this work, especially on the protein EspB, which has the correct dimensions and physicochemical properties to allow the transit of proteins through it, suggesting its participation in the outer-membrane machinery. Our work could serve as a thread to untangle the biggest mystery of the T7SS and provide the basis for a vaccine like the spike protein of SARS CoV2.