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Update on the virulence factors of the obligate pathogen *Mycobacterium tuberculosis* and related tuberculosis-causing mycobacteria

Jan MADACKI*, Guillem MAS FIOL** and Roland BROSCH*

Institut Pasteur, Unit for Integrated Mycobacterial Pathogenomics, CNRS UMR3525, Paris 75015, France

*For correspondence: Jan Madacki (jan.madacki@pasteur.fr) or Roland Brosch (roland.brosch@pasteur.fr)

** Present address: Institut Pasteur, Unité de Recherche Yersinia, Paris 75015, France

Abstract

Over the long course of evolution from a probable environmental reservoir, the pathogen that we know today as *Mycobacterium tuberculosis* has become fully capable of adapting to the life inside host cells by evading and modifying their responses to infection. Factors contributing to the success of this pathogen are numerous and thanks to a large body of work accumulated over the past decades, we are closer to understanding the remarkable complexity of tuberculosis pathogenesis. The unique type VII secretion systems and various complex lipids of the cell envelope have emerged as some of the most important and most studied factors in this regard. This review attempts to summarize recent findings on these and other virulence factors, while discussing their evolution in different closely related tuberculosis-causing bacteria as well, with the aim of exploring the processes which led *M. tuberculosis* to becoming one of the deadliest infections agents.

1 Introduction

With a death toll of estimated 1.3 million HIV-negative people and additional 374 000 deaths among HIV-positive people in the year 2016, tuberculosis (TB) is the leading cause of death by a single infectious agent worldwide (WHO, 2017). Its etiological agent, *Mycobacterium*

tuberculosis, is a primarily intracellular pathogen that is capable of successfully maintaining its viability within the host, involving a delicate interplay between the immune system and the pathogen's armaments, acquired and trained through thousands of years of host-pathogen interaction (Orgeur and Brosch, 2018). *M. tuberculosis* is spread by airborne transmission of bacteria-containing droplets from patients with active TB, allowing infection of lung alveoli in new hosts. After the initial stage of innate immune response, presentation of antigens by dendritic cells and recruitment of effector T-cells, characteristic multicellular structures called granulomas are formed with the aim of restricting the spread of the pathogen. In a weakened or immunocompromised host, the thereby established balance between pathogen and host responses may be lost, with subsequent systemic dissemination of the bacilli leading to active disease and new transmission. Besides *M. tuberculosis*, which is specialized on the human host, some highly related members (more than 99.99 % of genome sequence identity with *M. tuberculosis*) of the so-called *M. tuberculosis* complex (MTBC) can cause TB-like disease in other mammalian species, and most of these have kept their traditional names referring to the mammalian species from where they were isolated (*Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedii* etc.) and these members have been proposed to represent special ecotypes of the tubercle bacilli (Smith et al., 2009).

For studying mycobacterial pathogenesis on a cellular level, the use of macrophages has been proven as an invaluable tool, as bacteria are ingested primarily by these host cells upon entry into the alveoli, and events crucial to the disease process occur in their intracellular environment. Inside the infected macrophage, antibacterial mechanisms such as reactive oxygen, nitrogen intermediate production and phagolysosomal fusion are factors that usually function to eliminate pathogens. However, *M. tuberculosis* has evolved efficient ways to counteract some of these host-defense mechanisms. This also can be tested in selected cellular or animal infection models, mostly human or murine cell lines, as well as laboratory mice and guinea pigs, where the ability of a particular strain to multiply and cause lung and spleen pathology can be assessed and quantified over time.

Compared to some other bacterial pathogens, which may produce specific toxins or unique virulence factors, *M. tuberculosis* does not produce a single dominant factor responsible for causing disease, but rather induces pathology by a complex combination of virulence determinants and host responses. Extensive research on mycobacterial virulence, has helped to pinpoint several groups of factors involved in different stages of pathogenesis of TB. A key study of *in vivo* essentiality in the mouse model of infection using transposon site hybridization (TraSH) gave a first rough estimate of the global picture of *M. tuberculosis*

virulence factors, by identifying 194 *M. tuberculosis* genes as being specifically required for *in vivo* growth/survival in mice (Sassetti and Rubin, 2003). When a similar study was done on macrophages, 126 genes were identified, some of them being essential exclusively in the macrophage model (Rengarajan et al., 2005). Moreover, results of a more recent study using deep sequencing, significantly overlapped with the previous one from 2003, but found more than 400 additional genes as being essential *in vivo* (Zhang et al., 2013). These combined data now provide a framework for the definition of virulence genes of *M. tuberculosis*, whereby the individual genes may still be necessary to be studied in more detail to identify their exact roles and functions in the infection process. A number of genes identified as essential for virulence encode proteins involved in basic metabolism, a fact that makes defining a virulence factor in *M. tuberculosis* an arduous task. On the other hand, factors such as components of the ESX / type VII secretion systems, as well as several types of complex lipids of the cell envelope are standing at the frontline facing the host immune system and the most recent progress in the study of their biological roles and their participation in the virulence of *M. tuberculosis* constitutes the main focus of this review.

2 Type VII secretion systems

The *Mycobacterium tuberculosis* genome encodes five type VII secretion systems (ESX-1, ESX-2, ESX-3, ESX-4 and ESX-5) which are specialized for transport of selected protein substrates across the cell envelope (Abdallah et al., 2007; Brodin et al., 2004; Gröschel et al., 2016). Genes that likely encode core components of the secretion machinery (*ESX* conserved components, *Ecc*) are conserved in each of the five loci, with *esx-4* having the minimal set of genes encoding the simplest and earliest type VII secretion system, from which the rest probably originated via gene duplication and plasmid-mediated horizontal gene transfer (Dumas et al., 2016; Newton-Foot et al., 2016). A recently published structure of ESX-5 from *Mycobacterium xenopi* gives insight into the assembly of EccB, EccC, EccD and EccE into a functional complex (Fig. 1) (Beckham et al., 2017). ESX-1 emerged from several independent experimental approaches as one of the most significant for pathogenesis. Comparative genomic studies of *M. tuberculosis* with the attenuated vaccine strain *M. bovis* Bacille Calmette Guérin (BCG), as well as naturally attenuated *M. microti* have shown that parts of the ESX-1 secretion system are encoded in the regions of difference (RD) 1 (RD1^{BCG} and RD1^{mic}) (Brodin et al., 2002; Gordon et al., 1999; Mahairas et al., 1996). Both RD1^{BCG} and RD1^{mic} are notable for encoding secreted proteins ESAT-6 (early secretory antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa), also known as EsxA and EsxB,

respectively, which are known for a long time as potent T-cell antigens (Barnes et al., 1992; Berthet et al., 1998; Sørensen et al., 1995). By constructing an RD1 deletion mutant strain of *M. tuberculosis* H37Rv, as well as knock-in strains of *M. bovis* BCG and *M. microti* it was confirmed that both the secreted antigens, but also a functional secretion machinery are necessary for virulence (Hsu et al., 2003; Pym et al., 2002).

A considerable amount of research has been conducted in recent years attempting to clarify the fate of *M. tuberculosis* after infection. One of the main strategies of *M. tuberculosis* as an intracellular pathogen, after undergoing phagocytosis by the macrophages, is disabling or rather delaying the differentiation of the phagosome into a phagolysosome – thus avoiding the hostile acidic environment optimal for the activity of hydrolytic enzymes. Many factors have been implicated in this process and several pathogen strategies have been described, such as retention of vacuolar H⁺-ATPase (Queval et al., 2017; Wang et al., 2015; Wong et al., 2011), interfering with Rab GTPase recruitment (Via et al., 1997) and evading the toxicity of reactive oxygen and nitrogen species (Voskuil et al., 2011). Surprisingly, there were also indications that *M. tuberculosis* is capable of translocating from these endosomal compartments into the cytosol, probably at later stages of infection (McDonough et al., 1993; van der Wel et al., 2007). In a sophisticated assay using a fluorescent probe sensitive to the beta-lactamase activity localized on the surface of the bacterium and a fluorescence resonance energy transfer (FRET)-based method, contact of *M. tuberculosis* with the cytoplasm of macrophages was confirmed (Simeone et al., 2012). The ability of *M. tuberculosis* to rupture the phagosomal membrane was subsequently demonstrated *in vivo* in mouse lungs and by using an inhibitor of phagosomal maturation it was indicated that restriction of phagosomal acidification is a prerequisite of phagosomal rupture (Simeone et al., 2015). Most importantly, phagosomal rupture has been reproducibly linked to ESX-1, as neither *M. tuberculosis* Δ RD1 nor *M. bovis* BCG exerted this effect. The observation that ESX-1-deficient strains are unable to cause phagosomal rupture seem to be in concert with previously reported membrane lysing activity of EsxA (D. Houben et al., 2012; Hsu et al., 2003), although its precise function probably needs to be reexamined (Conrad et al., 2017). The consequences of cytosolic contact can be sensing of bacterial components, such as DNA via nucleotidyltransferase cGAS and by AIM-2, which results in type I interferon (IFN) production and activation the inflammasome, respectively (Collins et al., 2015; Wassermann et al., 2015; Watson et al., 2015). It should be mentioned however, that it is not clear from where the DNA that is sensed by cGAS specifically comes from. Apart from mycobacterial DNA that could potentially be released by the bacteria via membrane vesicles or yet unknown mechanisms (Majlessi et al., 2015), the

release of mitochondrial DNA into the cytosol has also been suggested (Wiens and Ernst, 2016). Lastly, cytosolic contact leads to cell death, as observed in different cellular infection models, promoting bacterial dissemination (Aguilo et al., 2013; Simeone et al., 2012; Augenstreich et al., 2017). Under *in vivo* conditions however, *M. tuberculosis*-infected macrophages tend to survive longer (Simeone et al., 2015), although it is tempting to speculate that phagosomal rupture and thereby induced host cell death might play a role during the formation of the caseous lesions inside granulomas, in which the tubercle bacilli find themselves in an extracellular environment.

M. bovis BCG is to date the only anti-TB vaccine strain licensed, although its efficacy for pulmonary TB in the adolescent and adult population is deficient, prompting urgent efforts for the development of refined or new vaccine strains. As a consequence of the RD1 deletion in BCG strains, phagosomal rupture and subsequent immune signaling events are absent, which seems to limit the protective efficacy of the vaccine. Hence, one of the approaches to develop a more efficient vaccine strain might involve engineering recombinant BCG strains with a functional ESX-1 system, as a BCG strain complemented with the extended RD1 region from *M. tuberculosis* H37Rv did improve this efficacy (Pym et al., 2003), but with safety concerns. As an alternative, a BCG strain harboring the ESX-1 locus of biosafety level 2 (BSL-2) species *Mycobacterium marinum* was prepared (Gröschel et al., 2017), providing desired improvement of protective efficacy in the murine model of infection, with functional phagosomal rupture inducing several host cell responses not present when using parental BCG, and at the same time showing strongly reduced virulence compared to *M. bovis* BCG ESX-1^{Mtb}. In parallel, reactogenicity to ESX-1-secreted antigens that are absent from BCG has also been suggested to be linked to the improved protection against *M. tuberculosis* of other vaccine strains, such as the attenuated *M. tuberculosis* vaccine MTBVAC (Aguilo et al., 2017), which is presently in late clinical development as a live vaccine (Spertini et al., 2015).

Alongside the ESX-1 secretion system, the ESX-5 and ESX-3 systems have also been implicated in virulence of pathogenic mycobacteria. ESX-5 is the most recently evolved type VII secretion system and can be found only in slow growing mycobacterial species (Gey van Pittius et al., 2001). Initially revealed in *Mycobacterium marinum* (Abdallah et al., 2009), the major function of the ESX-5 secretion system appears to be secretion of substrates belonging mostly to the unique families of PE and PPE proteins named after their N-terminal Pro-Glu and Pro-Pro-Glu motifs, respectively (Cole et al., 1998). These large and somewhat mysterious families of proteins comprising about 8 % of the genome of *M. tuberculosis* H37Rv are further divided into subgroups, depending on motifs present in their C-terminal

sequence (Cole et al., 1998). The emergence of PE polymorphic GC-rich-repetitive sequence (PE_PGRS) and the PPE major polymorphic tandem repeat (PPE-MPTR) subfamilies represents the most recent evolutionary event among the mycobacterial PE/PPE sequences, and these are thus present only in pathogenic species (Gey van Pittius et al., 2006). Indications that the ESX-5 system is implicated in pathogenesis initially came from research on *M. marinum* (Abdallah et al., 2008; Ramakrishnan et al., 2000; Weerdenburg et al., 2012). Subsequent characterization of different ESX-5 mutants of *M. tuberculosis* showed that inactivation of the ESX-5 system resulted in a defect in PPE protein export, impaired cell-wall integrity and strong attenuation (Bottai et al., 2012) as well as defects in PE_PGRS export (E. N. G. Houben et al., 2012). In addition, these experiments also revealed that certain ESX-5 components were essential for *in vitro* growth of *M. tuberculosis*, as certain ESX-5 knock-out mutants could only be obtained when a second copy of selected ESX-5 genes was introduced (Di Luca et al., 2012) or if the cell wall composition was weakened (Ates et al., 2015). Moreover, a mutant in *esx-5* region-encoded *ppe/pe* genes *M. tuberculosis* $\Delta ppe25-pe19$ has shown attenuation, but still was able to induce strong CD4⁺ T-cell immunity owing to cross-reactivity with numerous PE/PPE homologs encoded by other parts of the genome (Sayes et al., 2016, 2012). While the molecular mechanism underlying the attenuation of the $\Delta ppe25-pe19$ construct remains currently unknown, the interference with the export of certain PE and PPE proteins has also been associated with increase of virulence of selected *M. tuberculosis* strains. In this respect, interesting observations were made for *M. tuberculosis* strains that had mutated or lost the gene *ppe38*, whose presence was found to be required for ESX-5-mediated secretion of more than 80 PE_PGRS and PPE-MPTR substrates. In the same study it was also found that the *ppe38* locus was deleted through IS6110-mediated recombination processes (Gonzalo-Asensio et al., 2018) in most *M. tuberculosis* strains of the so-called “Beijing” family (lineage 2), raising important questions on the role of these PE_PGRS and PPE_MPTR proteins in virulence, but also on the mechanism of their secretion (Ates et al., 2018b). Moreover, the *ppe38* locus is also deleted from a series of animal-adapted members of the MTBC, such as *M. bovis*, due to deletion of the RD5 region. Consequently, also the vaccine strain BCG Pasteur does not export the plethora of PE_PGRS and PPE_MPTR proteins, a phenotype which could be repaired by genetic complementation with a copy of the *ppe38* locus from *M. tuberculosis* in recombinant BCG38 (Ates et al., 2018c). Interestingly, despite the restauration of the PE_PGRS and PPE-MPTR export in such recombinant BCG38 strain, vaccination trials in different mouse infection models have not shown a significant change in the vaccine potential of the recombinant strain, questioning somewhat the specificity of some

of the PPE-MPTR protein components of subunit booster vaccines tested in clinical vaccination trials (Ates et al., 2018c).

Investigated to a lesser extent, ESX-3 has been implicated in virulence, aside from its role in iron and zinc acquisition (Serafini et al., 2009; Siegrist et al., 2014) through several PE/PPE proteins (Tufariello et al., 2016) and its secreted substrates, EsxH and EsxG were also found to inhibit phagosomal maturation (Mehra et al., 2013; Portal-Celhay et al., 2017).

3 Other surface exposed factors

Other components of the cell envelope of *M. tuberculosis* have emerged as important virulence factors, based on their strategic location on the host-pathogen interface. The mycobacterial cell envelope has a complex organization (Brennan and Nikaido, 1995) and consists of a conventional plasma membrane, a layer of peptidoglycan with a covalently attached polysaccharide named arabinogalactan which has its pentaarabinosyl ends esterified by mycolic acids. These latter components, which are characteristic of mycobacteria and associated to the name of this bacterial genus, are very long, 60-90 carbon-atom containing α -alkyl β -hydroxy fatty acids which are essential for viability of the mycobacterial cell (Grzegorzewicz et al., 2012a; Portevin et al., 2005; Vilch  ze et al., 2000). These mycolic acid residues are the main components of the inner leaflet of the mycobacterial outer membrane, also called the mycomembrane – a unique lipid bilayer, with the outer leaflet consisting of various types of non-covalently bound lipids, such as trehalose mono- and dimycolates (TMM, TDM), phthiocerol dimycocerosates (PDIM), di- and polyacyl trehaloses (DAT, PAT) and sulfolipids (SL), some of which are transported by mycobacterial transporters of the MmpL family to the outer membrane, thereby constituting targets for drug development (Degiacomi et al., 2017; Grzegorzewicz et al., 2012b; Viljoen et al., 2017). The richness of structures of mycobacterial cell envelope lipids has been a subject of research for decades. Some of these lipids are found throughout the *Mycobacterium* genus, such as trehalose mono- and dimycolates, and some, notably phthiocerol dimycocerosates (abbreviated PDM (Brennan and Nikaido, 1995), DIM (Augenstreich et al., 2017) or PDIM (Quigley et al., 2017)) are present only in certain mycobacterial species.

Based on research accumulated over the decades, it became clear that mycobacterial surface lipids and glycoconjugates are important factors for entry into macrophages, stimulating various receptors. As early as the 1950s, TDM has been shown to be an important effector molecule, also receiving the name “cord factor”, as it was thought to be the only factor responsible for the characteristic cord-like appearance of *M. tuberculosis* in

microscopic preparations (Bekierkunst, 1968; Bloch and Noll, 1955). By stimulating the macrophage inducible C-type lectin (Mincle), TDM causes induction of cytokine and nitric oxide production (Ishikawa et al., 2009) and at the same time TDM may be one of the factors contributing to phagosome maturation delay, which was shown by using TDM-coated beads (Indrigo et al., 2003). TDM is an abundant cell-wall component of all bacteria belonging to the *Mycobacterium* genus, but subtle modifications of mycolic acid residues, such as cyclopropyl groups which are normally present only in pathogenic species (Barry et al., 1998) (Fig. 2B, C) might be an important factor. Immunomodulatory significance of mycolic acid modifications was confirmed in a laborious study of constructing an *M. tuberculosis* strain lacking all *S*-adenosylmethionine dependent methyltransferases responsible for cyclopropyl group modifications of mycolates and a strain additionally lacking oxygenated functional groups (Barkan et al., 2012). Results of using these strains in a murine model of infection show severe attenuation and changes in antigen-specific immune response dependent on the presence of specific modifications. Deletion of the *hma* gene, which is essential for synthesis of both oxygenated mycolate types (methoxy- and ketomycolates) shows that these forms contribute to cell wall integrity, but also seem to be important for specifically inhibiting IL-12p40 mediated responses (Dao et al., 2008; Dubnau et al., 2000). Considering the complexity of the biosynthesis of mycolic acids and the apparent interconnectedness of the enzymes in this pathway by protein-protein interactions (Cantaloube et al., 2011; Veyron-Churlet et al., 2005), mutations in genes encoding core components of the fatty acid synthase system II (FASII), such as *kasB* and *hadC* (Fig. 2A) seem to affect the mycolic acid composition and, consequently, the virulence of respective strains (Bhatt et al., 2007; Slama et al., 2016). Oxygenated mycolates, in particular, are crucial for forming so-called foamy macrophages – differentiated infected macrophages rich in lipid droplets which enable the persistence of the bacilli (Dkhar et al., 2014; Peyron et al., 2008) and recent steps in elucidating the biosynthesis and metabolism of these mycolate classes (Madacki et al., 2018) could provide new clues for studying their impact on the virulence and persistence of mycobacteria.

Contrary to trehalose mycolates, which are common lipids of the mycobacterial cell wall, other identified trehalose containing lipids – di- and polyacyl trehaloses (DAT, PAT) are found exclusively in members of the *M. tuberculosis* complex, and sulfolipids (SL) were suggested to be produced only in *M. tuberculosis* (Daffé and Draper, 1997). The acyl chains of these lipids are synthesized by specialized polyketide synthase systems (PKS), resulting in polymethylated hydrocarbon chains. The specific occurrence of these lipids in pathogenic

strains has naturally raised a question of their involvement in tuberculosis pathogenesis, with rather ambiguous answers when using PKS mutants *in vivo* (Rousseau et al., 2003b, 2003a). A recent study analyzed multiple deletion strains deficient in DAT, PAT and SL synthesis, but also in PDIM, which contain methyl branched acyl chains, with the conclusion that all of the mentioned lipids contribute to phagosome maturation arrest, but with PDIM having a more dominant role in this process, thus masking the effect of the former (Passemar et al., 2014).

The role of sulfolipids in virulence has been questioned previously, notably by Rousseau et al., 2003b. However, the production of SL was more recently linked to the PhoP/R two component regulatory system, suggesting that PhoP-deficient strains have no or very low expression of genes encoding for proteins involved in SL biosynthesis (Gonzalo-Asensio et al., 2014), with possible implication on virulence. Additionally, using an approach aimed at identifying factors modulating NF- κ B-dependent signaling, a transposon mutant defective in SL biosynthesis was identified, showing that SL are Toll-like receptor 2 antagonists (Blanc et al., 2017). Methyl-branched fatty acid-containing lipids of *M. tuberculosis* apparently have a buffering role, as polyketide synthases incorporate toxic propionate, which comes from cholesterol catabolism, a significant energy source for the intracellular bacilli (Lee et al., 2013). Apart from their immunogenic properties, PDIM were shown to have an important structural role as components of the mycomembrane, but it was also suggested that their incorporation into the host plasma membrane is able to alter its biophysical properties with downstream effects on phagocytic receptors (Astarie-Dequeker et al., 2009; Camacho et al., 2001). Furthermore, it was shown that PDIM are indispensable for efficient phagosomal rupture mediated by ESX-1, a remarkable example of coordinated usage of proteins and lipids, which awaits more mechanistic insights (Augenstreich et al., 2017; Quigley et al., 2017). Another group of polymethylated acyl chain containing molecules found in mycobacteria are lipooligosaccharides (LOS), which are, however absent in *M. tuberculosis*. Interestingly, these surface molecules are present in *Mycobacterium canettii* strains, which bare the closest resemblance to the progenitor of *M. tuberculosis* (Supply et al., 2013; Boritsch et al., 2016b). It was recently ascertained that LOS are responsible for the characteristic smooth colony morphology of *M. canettii* strains, owing to the presence of an intact dual *pks5* locus, which has apparently undergone homologous recombination resulting in loss of selected *pks5* functions and *pap*-encoded acyltransferase activity in *M. tuberculosis* during its evolution into a strict pathogen (Boritsch et al., 2016a). This recombination event might have resulted in an evolutionary advantage for *M. tuberculosis*, judging from the

increased fitness and virulence of spontaneous rough morphotype and LOS-deficient *M. canettii* K compared to its smooth counterpart. Another group of PKS-synthesized lipids, phenolic glycolipids (PGL), is found only in a subset of *M. tuberculosis* strains, such as the hypervirulent Beijing lineage, due to a frameshift mutation in the *pks15/I* gene (Constant et al., 2002). Although immunomodulatory activity of PGLs from *M. tuberculosis* has been described (Reed et al., 2004), their reported contribution to the hypervirulent phenotype of the Beijing strains is not fully understood (Huet et al., 2009; Sinsimer et al., 2008).

The abundant mannosylated components of the cell envelope of mycobacteria – phosphatidylinositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) were extensively studied for their importance in host-cell recognition – see (Vergne et al., 2015) for a recent review. Variations in lipoarabinomannan structure and their impact on its immunogenic properties *in vitro* have attracted much attention, notably the pathogenic species-specific mannose cap motif, resulting in ManLAM, which was shown to contribute to phagosome maturation arrest (Chatterjee et al., 1992; Fratti et al., 2003). An additional, and unusual, methylthio-D-xylose motif was also characterized in *M. tuberculosis*, binding to one mannose cap per molecule of ManLAM (Joe et al., 2006; Treumann et al., 2002), while a recently discovered genetic locus responsible for its biosynthesis will enable elucidation of its biological role (Angala et al., 2017). Mutant strains of *M. bovis* BCG and *M. tuberculosis* producing capless LAM, however, failed to show changes in *in vivo* replication and cytokine production, rather suggesting a redundant function (Afonso-Barroso et al., 2012) which prompts deeper research into the biological functions of this important glycoconjugate.

Although the protein composition of the mycomembrane is still largely unexplored, interesting insights are beginning to emerge from their studies. One of the few characterized integral proteins of the mycomembrane, CpnT is a channel forming protein with an N-terminal exotoxin domain, which confers the ability of *M. tuberculosis* to induce necrosis of infected cells (Danilchanka et al., 2014; Sun et al., 2015). Upon exerting its necrotizing activity, CpnT does not appear to induce vast cytokine production, and is thus suggested to allow “quiet” cellular escape and dissemination of the bacilli (Maueröder et al., 2016). Alternative approaches for dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane in rapidly growing mycobacteria have suggested that the outer membrane fraction might also contain Antigen 85 complex proteins (see below) and members of the large Mce (mammalian cell entry) protein family (Chiaradia et al., 2017). Finally, other potential candidates that might be localized in the outer membrane are the EspACD proteins, which are associated to the ESX-1 type VII system (Orgeur and Brosch,

2018) (Fig. 1). EspC, for example was shown to form a filamentous structure in the mycobacterial cell envelope, which could theoretically be embedded in the mycomembrane layer (Lou et al., 2017).

Several important secreted proteins independent from type VII secretion systems (Fig. 1) have also been described as virulence factors. For example, the three mycolyl transferases that are exported by the Twin- arginine translocation (TAT) system catalyze the attachment of mycolate residues onto arabinogalactan, as well as the synthesis of TDM (Belisle et al., 1997; Jackson et al., 1999; Katti et al., 2008). These major secreted immunogens also known as the Antigen 85 complex, are also found associated to the mycobacterial cell surface (Wiker and Harboe, 1992). These antigens have previously been associated to altered vaccination efficacy (Horwitz et al., 1995; Ndiaye et al., 2015), but more recent studies have found that the level of export and the availability of the Ag85 proteins for generating immune responses is influenced by the PhoP/R two component regulatory system (Sayes et al., 2018; Solans et al., 2014), and the use of Ag85 as immunogens might not be generally applicable for protection against all *M. tuberculosis* strains, as important, strain lineage-dependent variation of immune recognition might exist (Sayes et al., 2018). Another notable example is the protein tyrosine phosphatase PtpA, directly binding to vacuolar H⁺-ATPase, thus being one of the main inhibitors of phagosome acidification (Wong et al., 2011). However, as mentioned before, the inhibition of phagosomal acidification and maturation by mycobacteria seems to be more complex, like recently found by the screening of host-factors that impact this process (Queval et al., 2017) as well as the identification of specific proteins (SapM, PknG) that are exported via the SecA2 mediated general secretory pathway (GSP) (Fig. 1) and also impact acidification/maturation of mycobacteria-containing phagosomes (Zulauf et al., 2018).

4 Miscellaneous factors

A considerable number of factors that were found to be indispensable for proper process of pathogenesis do not necessarily fall into the category of surface-exposed effectors directly interacting with the host. The above-mentioned preferential utilization of host-cell cholesterol, but also triacylglycerols by intracellular *M. tuberculosis* relies on several enzymes required for their degradation, their absence causing severe attenuation (Crowe et al., 2018, 2017; Daniel et al., 2011; Pandey and Sasseti, 2008; Singh et al., 2017; VanderVen et al., 2015). In this context, members of the Mce family of proteins should again be mentioned, as they are thought to import cholesterol or other hydrophobic substrates such as fatty acids and were shown to be implicated in virulence of tubercle bacilli (Marjanovic et al., 2010; Nazarova et

al., 2017; Senaratne et al., 2008; Shimono et al., 2003; Ekiert et al., 2017). Also, research of gene and posttranslational regulation has also pointed out the importance of DosR, WhiB, PhoP (Geiman et al., 2006; Smith et al., 2017), adenylyl cyclases (Samanta et al., 2017; Shleeve et al., 2017), and protein kinase *pknG* (Khan et al., 2017; Rieck et al., 2017), and new insights on manipulation of small molecules interfering with cytosolic surveillance are emerging (Dey et al., 2015, 2017). Finally, in our selection of recently identified and/or confirmed virulence factors of *M. tuberculosis* that are presented in this review, we also would like to highlight a dedicated gene cluster, named *moaAI-DI*. The proteins encoded by this cluster have been recently found to enable the tubercle bacillus to respire nitrate and to survive oxygen depletion, a feature which seems particularly important in hypoxic granulomas in the host (Levillain et al., 2017). Strikingly, this gene cluster, which is involved in the biosynthesis of the molybdenum cofactor seems to have been acquired horizontally together with its hypoxia-responsive transcriptional regulator by a recent common ancestor of the tubercle bacilli from plasmids of environmental bacteria of another phylum (β -proteobacteria *Burkholderia vietnamiensis*) (Levillain et al., 2017) (Fig. 3A). The specific acquisition by tubercle bacilli is underlined by the absence of the *moaAI-DI* cluster in closely related mycobacteria (*M. marinum*, *Mycobacterium kansasii*) (Fig. 3B) that have been used as model organisms for studying the evolution and emergence of *M. tuberculosis* (Stinear et al., 2008; Wang et al., 2015). Apart from the MTBC strains, the *moaAI-DI* genetic locus is also present in *M. canettii* strains, although it is not complete in all the strains (<http://genolist.pasteur.fr/CanettiiList/>), which suggests that after the acquisition of the *moaAI-D* region via horizontal transfer, parts of the locus were lost in a subgroup of *M. canettii* strains. However, in the MTBC this locus is highly conserved, suggesting that the associated function in nitrate respiration (Williams et al., 2014) is important and a matter of selection, apparently giving MTBC members some advantages for the survival in mammalian hosts (Levillain et al., 2017).

In conclusion, as briefly shown in this review by selected examples, the evolution of *M. tuberculosis* towards pathogenicity was a complex process involving a multitude of adaptative steps, some of which appear ancient while others are likely more recent. *M. tuberculosis* is a highly adapted, professional human pathogen which needs to cause disease in the human lungs for efficient spread. Thus, the co-evolution of humans and *M. tuberculosis* has resulted in an intimate host-pathogen relationship. How long it really has been, this is a question of debate, estimations range from 70000 years (Comas et al., 2013) to 6000 years (Bos et al.,

2014), whereby the latter hypothesis becomes more and more accepted as it is based on the analysis of ancient DNA samples and less on estimations derived from genome comparisons of extant mycobacterial strains (Kay et al., 2015; Mokrousov, et al., 2017). However, it should also be repeated here that the last common ancestor of the MTBC was likely a strain that resembled *M. canettii*, which underwent a phenomenal clonal expansion by becoming a dedicated pathogen. It is well known that the MTBC besides *M. tuberculosis* also harbors very closely related relatives that infect selected other mammalian species than humans as their preferential hosts. Interestingly, these animal-adapted species seem to have evolved from a sublineage of the *M. tuberculosis* complex, which is characterized by the deletion of the region RD9 (Brosch et al., 2002; Orgeur and Brosch, 2018), and also comprises the two *M. africanum* lineages L5 and L6, which cause tuberculosis in humans in West Africa (Ates et al., 2018a; de Jong et al., 2010; Otchere et al., 2018). As such, *M. africanum* strains seem to be closely related to the common ancestor of animal strains and it will be of particular interest to determine which molecular factors have been involved in the adaptation processes of these tubercle bacilli to new animal hosts (Gonzalo-Asensio et al., 2014; Malone et al., 2018; Orgeur and Brosch, 2018). One surprising feature in this respect is the long-time known finding that *M. tuberculosis* in contrast to *M. bovis* does not cause disease in cattle, which was recently re-established in an advanced cattle infection model (Villarreal-Ramos et al., 2018). These are just another few examples of how complex the relationship between tubercle bacilli and their various hosts are. Similar processes have certainly also contributed to the emergence of *M. tuberculosis* strains from probably less virulent and much less prevalent ancestor pools (resembling *M. canettii* strains) into the professional human pathogens that they represent today. Without doubt, such factors make up the highly complex mosaic scaffold of mycobacterial virulence, for which we have tried to highlight some of the key players that we consider having an important impact on virulence. This said, there is certainly much more room for studying further virulence factors of *M. tuberculosis*, as well as host immunity factors that have not been covered in this review, but which can also contribute strongly to advance or restrict disease progression, and thereby influence the outcome of infection. The particularities of TB as disease in relation to the host and the extremely wide, and often specific distribution of certain *M. tuberculosis* strain families in the global human population make the infection dynamics associated to TB very complex. Research on TB-causing mycobacterial pathogens will therefore need to continue for a better understanding of the evolutionary adaptation processes that made *M. tuberculosis* such a powerful and devastating pathogen and for finding new possible solutions to counteract this situation.

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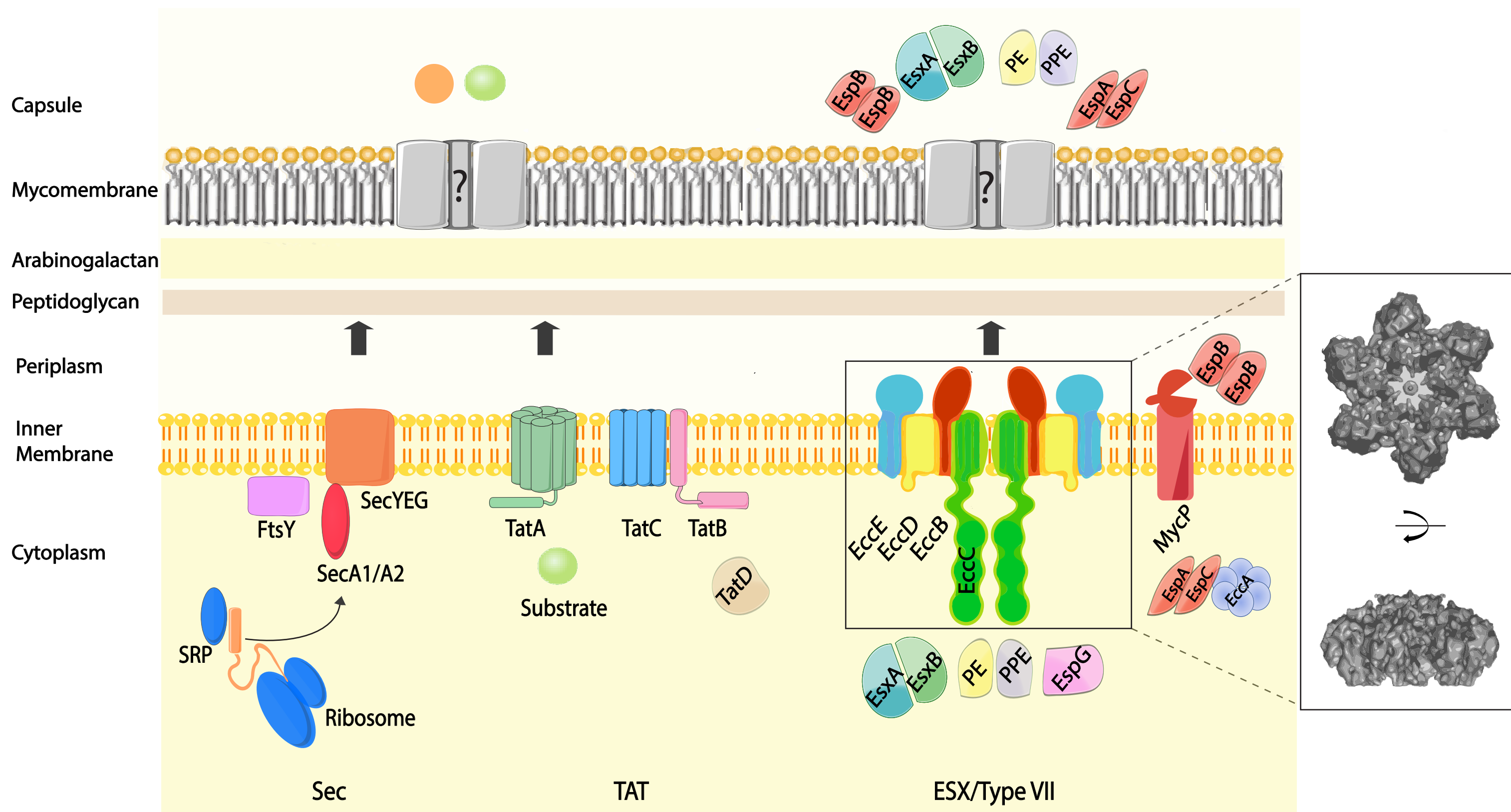
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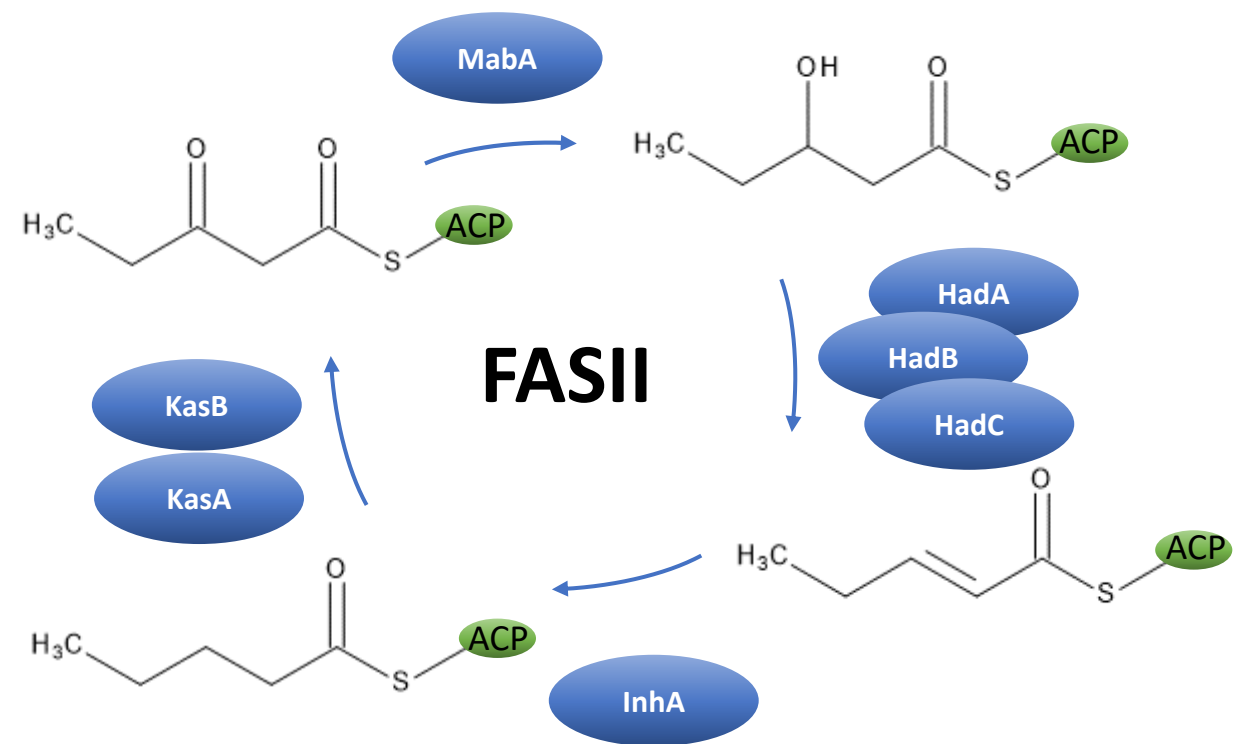
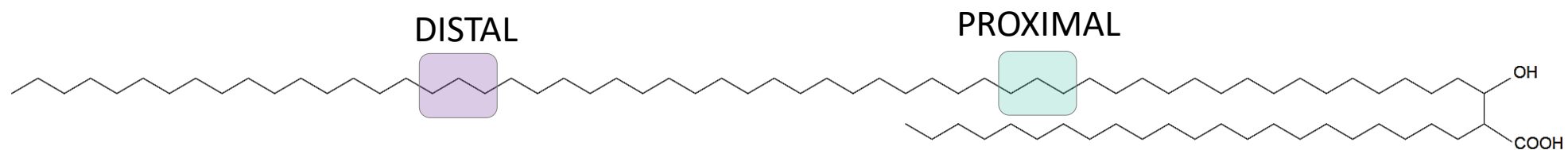
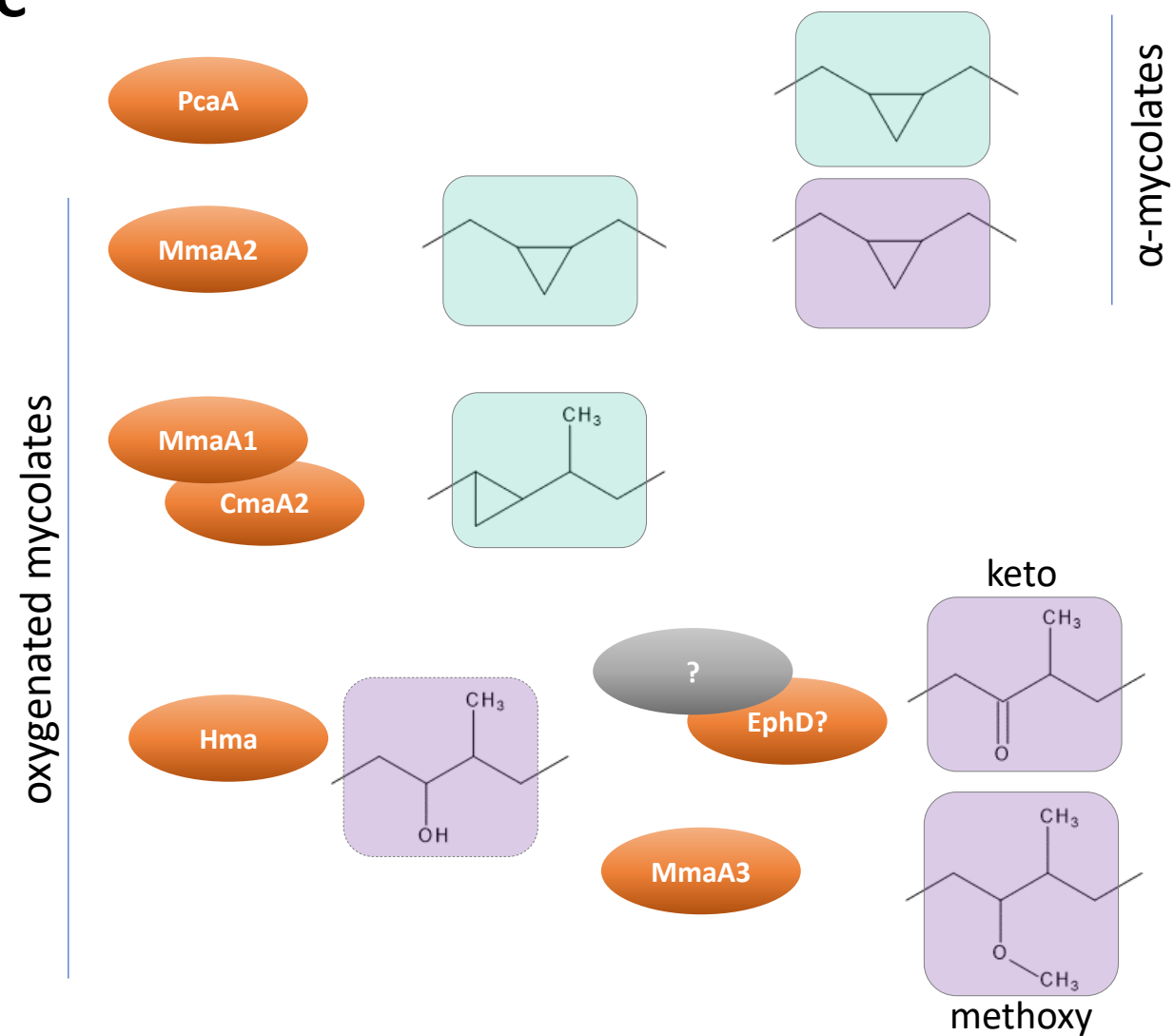
Figure 1: Schematic representation of three types of secretion systems present in mycobacteria – the general Sec secretory pathway, the TAT pathway and the ESX / type VII secretion systems. The three-dimensional structure of ESX-5 system from *Mycobacterium xenopi* as determined by electron microscopy and single particle analysis (Beckham et al., 2017) is also shown.

Figure 2: Functional groups are attached to the main (meromycolic) hydrocarbon chain of mycolic acids likely during its elongation by fatty acid synthase system II (FASII) (A) in two distinct positions relative to the carboxyl group (B) – distal and proximal – resulting in dicyclopropylated α -mycolates and oxygenated methoxy- and ketomycolates (C). The mycolic acid modifying enzymes (in orange) are connected to the core enzymes of the FASII system (in blue) through protein-protein interactions.

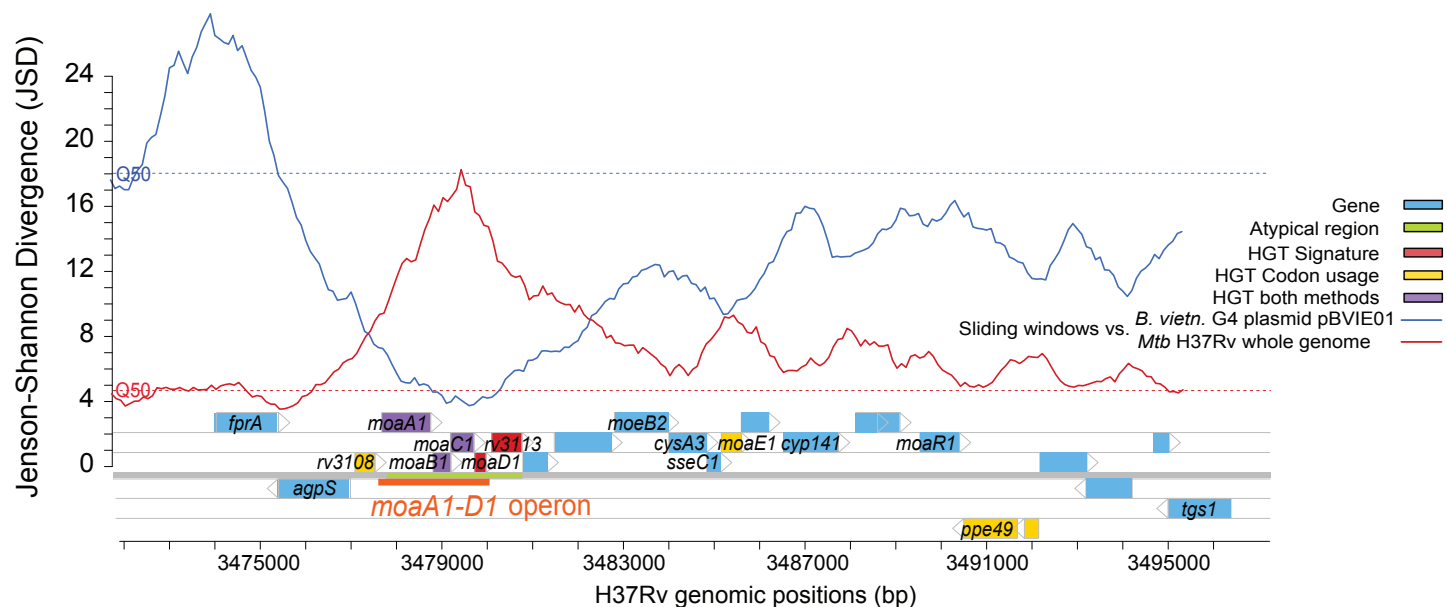
Figure 3: Example of a genomic locus (*moaA1-D1* locus) acquired by tuberculosis-causing mycobacteria: (A) Jensen-Shannon divergence profiles of the *M.tuberculosis* genomic region harboring the Moco-1 island and its genomic context. The red curve represents the divergence between the signature of the whole *M. tuberculosis* H37Rv genome and that of the sliding windows (5-kb long, 100-bp step) over the region. The blue curve represents the divergence between the signature of the *Burkholderia vietnamiensis* G4 plasmid pBVIE01 and that of the sliding windows over the region, as described in detail by Levillain et al., 2017. Image adapted from Ref.(Levillain et al., 2017); (B) genomic region of the horizontally transferred *moaA1-D1* locus in *M. tuberculosis* and its flanking regions containing conserved mycobacterial genes in comparison to the orthologous regions in *Mycobacterium marinum* M and *Mycobacterium kansasii* ATCC 12478. Note that *M. marinum* M shows the minimal gene content in this genomic region, while in *M. tuberculosis* the *moaA1-D1* gene cluster showing similarity to the *B. vietnamiensis* G4 plasmid pBVIE01 encoded genes has been inserted in addition to mycobacterial genes involved in molybdenum cofactor biosynthesis. In *M. kansasii* ATCC 12478, genes with different functions, including one encoding a putative polyketide synthase, are present in the genomic locus. Percentages on grey lines represent amino acid identity values between gene products of respective strains. Color coding of genes refers to the scheme used in TubercuList or CanettiiList

1161 (<http://genolist.pasteur.fr/CanettiiList/>). *Mtb* = *M. tuberculosis*; vs. = versus; *B. vientn.* =
1162 *Burkholderia vietnamiensis*; HGT = Horizontal Gene Transfer.



A**B****C**

A



B

