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

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5 Jan MADACKI*, Guillem MAS FIOL** and Roland BROSCHE*

6
7 Institut Pasteur, Unit for Integrated Mycobacterial Pathogenomics, CNRS UMR3525, Paris
8 75015, France

9
10
11 *For correspondence: Jan Madacki (jan.madacki@pasteur.fr) or Roland Brosch
12 (roland.brosch@pasteur.fr)

13 ** Present address: Institut Pasteur, Unité de Recherche Yersinia, Paris 75015, France
14

15
16 **Abstract**

17
18 Over the long course of evolution from a probable environmental reservoir, the pathogen that
19 we know today as *Mycobacterium tuberculosis* has become fully capable of adapting to the
20 life inside host cells by evading and modifying their responses to infection. Factors
21 contributing to the success of this pathogen are numerous and thanks to a large body of work
22 accumulated over the past decades, we are closer to understanding the remarkable complexity
23 of tuberculosis pathogenesis. The unique type VII secretion systems and various complex
24 lipids of the cell envelope have emerged as some of the most important and most studied
25 factors in this regard. This review attempts to summarize recent findings on these and other
26 virulence factors, while discussing their evolution in different closely related tuberculosis-
27 causing bacteria as well, with the aim of exploring the processes which led *M. tuberculosis* to
28 becoming one of the deadliest infections agents.

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31
32 **1 Introduction**

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

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

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

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

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

85

86 **2 Type VII secretion systems**

87 The *Mycobacterium tuberculosis* genome encodes five type VII secretion systems (ESX-1,
88 ESX-2, ESX-3, ESX-4 and ESX-5) which are specialized for transport of selected protein
89 substrates across the cell envelope (Abdallah et al., 2007; Brodin et al., 2004; Gröschel et al.,
90 2016). Genes that likely encode core components of the secretion machinery (*ESX* conserved
91 components, *Ecc*) are conserved in each of the five loci, with *esx-4* having the minimal set of
92 genes encoding the simplest and earliest type VII secretion system, from which the rest
93 probably originated via gene duplication and plasmid-mediated horizontal gene transfer
94 (Dumas et al., 2016; Newton-Foot et al., 2016). A recently published structure of ESX-5 from
95 *Mycobacterium xenopi* gives insight into the assembly of EccB, EccC, EccD and EccE into a
96 functional complex (Fig. 1) (Beckham et al., 2017). ESX-1 emerged from several independent
97 experimental approaches as one of the most significant for pathogenesis. Comparative
98 genomic studies of *M. tuberculosis* with the attenuated vaccine strain *M. bovis* Bacille
99 Calmette Guérin (BCG), as well as naturally attenuated *M. microti* have shown that parts of
100 the ESX-1 secretion system are encoded in the regions of difference (RD) 1 (RD1^{BCG} and
101 RD1^{mic}) (Brodin et al., 2002; Gordon et al., 1999; Mahairas et al., 1996). Both RD1^{BCG} and
102 RD1^{mic} are notable for encoding secreted proteins ESAT-6 (early secretory antigenic target of
103 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa), also known as EsxA and EsxB,

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

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

138 release of mitochondrial DNA into the cytosol has also been suggested (Wiens and Ernst,
139 2016). Lastly, cytosolic contact leads to cell death, as observed in different cellular infection
140 models, promoting bacterial dissemination (Aguilo et al., 2013; Simeone et al., 2012;
141 Augenstreich et al., 2017). Under *in vivo* conditions however, *M. tuberculosis*-infected
142 macrophages tend to survive longer (Simeone et al., 2015), although it is tempting to
143 speculate that phagosomal rupture and thereby induced host cell death might play a role
144 during the formation of the caseous lesions inside granulomas, in which the tubercle bacilli
145 find themselves in an extracellular environment.

146 *M. bovis* BCG is to date the only anti-TB vaccine strain licensed, although its efficacy
147 for pulmonary TB in the adolescent and adult population is deficient, prompting urgent efforts
148 for the development of refined or new vaccine strains. As a consequence of the RD1 deletion
149 in BCG strains, phagosomal rupture and subsequent immune signaling events are absent,
150 which seems to limit the protective efficacy of the vaccine. Hence, one of the approaches to
151 develop a more efficient vaccine strain might involve engineering recombinant BCG strains
152 with a functional ESX-1 system, as a BCG strain complemented with the extended RD1
153 region from *M. tuberculosis* H37Rv did improve this efficacy (Pym et al., 2003), but with
154 safety concerns. As an alternative, a BCG strain harboring the ESX-1 locus of biosafety level
155 2 (BSL-2) species *Mycobacterium marinum* was prepared (Gröschel et al., 2017), providing
156 desired improvement of protective efficacy in the murine model of infection, with functional
157 phagosomal rupture inducing several host cell responses not present when using parental
158 BCG, and at the same time showing strongly reduced virulence compared to *M. bovis* BCG
159 ESX-1^{Mtb}. In parallel, reactogenicity to ESX-1-secreted antigens that are absent from BCG
160 has also been suggested to be linked to the improved protection against *M. tuberculosis* of
161 other vaccine strains, such as the attenuated *M. tuberculosis* vaccine MTBVAC (Aguilo et al.,
162 2017), which is presently in late clinical development as a live vaccine (Spertini et al., 2015).

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

172 sequence (Cole et al., 1998). The emergence of PE polymorphic GC-rich-repetitive sequence
173 (PE_PGRS) and the PPE major polymorphic tandem repeat (PPE-MPTR) subfamilies
174 represents the most recent evolutionary event among the mycobacterial PE/PPE sequences,
175 and these are thus present only in pathogenic species (Gey van Pittius et al., 2006).
176 Indications that the ESX-5 system is implicated in pathogenesis initially came from research
177 on *M. marinum* (Abdallah et al., 2008; Ramakrishnan et al., 2000; Weerdenburg et al., 2012).
178 Subsequent characterization of different ESX-5 mutants of *M. tuberculosis* showed that
179 inactivation of the ESX-5 system resulted in a defect in PPE protein export, impaired cell-wall
180 integrity and strong attenuation (Bottai et al., 2012) as well as defects in PE_PGRS export (E.
181 N. G. Houben et al., 2012). In addition, these experiments also revealed that certain ESX-5
182 components were essential for *in vitro* growth of *M. tuberculosis*, as certain ESX-5 knock-out
183 mutants could only be obtained when a second copy of selected ESX-5 genes was introduced
184 (Di Luca et al., 2012) or if the cell wall composition was weakened (Ates et al., 2015).
185 Moreover, a mutant in *esx-5* region-encoded *ppe/pe* genes *M. tuberculosis* $\Delta ppe25-pe19$ has
186 shown attenuation, but still was able to induce strong CD4⁺ T-cell immunity owing to cross-
187 reactivity with numerous PE/PPE homologs encoded by other parts of the genome (Sayes et
188 al., 2016, 2012). While the molecular mechanism underlying the attenuation of the $\Delta ppe25-$
189 $pe19$ construct remains currently unknown, the interference with the export of certain PE and
190 PPE proteins has also been associated with increase of virulence of selected *M. tuberculosis*
191 strains. In this respect, interesting observations were made for *M. tuberculosis* strains that had
192 mutated or lost the gene *ppe38*, whose presence was found to be required for ESX-5-mediated
193 secretion of more than 80 PE_PGRS and PPE-MPTR substrates. In the same study it was also
194 found that the *ppe38* locus was deleted through IS6110-mediated recombination processes
195 (Gonzalo-Asensio et al., 2018) in most *M. tuberculosis* strains of the so-called “Beijing”
196 family (lineage 2), raising important questions on the role of these PE_PGRS and PPE_MPTR
197 proteins in virulence, but also on the mechanism of their secretion (Ates et al., 2018b).
198 Moreover, the *ppe38* locus is also deleted from a series of animal-adapted members of the
199 MTBC, such as *M. bovis*, due to deletion of the RD5 region. Consequently, also the vaccine
200 strain BCG Pasteur does not export the plethora of PE_PGRS and PPE_MPTR proteins, a
201 phenotype which could be repaired by genetic complementation with a copy of the *ppe38*
202 locus from *M. tuberculosis* in recombinant BCG38 (Ates et al., 2018c). Interestingly, despite
203 the restauration of the PE_PGRS and PPE-MPTR export in such recombinant BCG38 strain,
204 vaccination trials in different mouse infection models have not shown a significant change in
205 the vaccine potential of the recombinant strain, questioning somewhat the specificity of some

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

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

212

213 **3 Other surface exposed factors**

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

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

240 microscopic preparations (Bekierkunst, 1968; Bloch and Noll, 1955). By stimulating the
241 macrophage inducible C-type lectin (Mincle), TDM causes induction of cytokine and nitric
242 oxide production (Ishikawa et al., 2009) and at the same time TDM may be one of the factors
243 contributing to phagosome maturation delay, which was shown by using TDM-coated beads
244 (Indrigo et al., 2003). TDM is an abundant cell-wall component of all bacteria belonging to
245 the *Mycobacterium* genus, but subtle modifications of mycolic acid residues, such as
246 cyclopropyl groups which are normally present only in pathogenic species (Barry et al., 1998)
247 (Fig. 2B, C) might be an important factor. Immunomodulatory significance of mycolic acid
248 modifications was confirmed in a laborious study of constructing an *M. tuberculosis* strain
249 lacking all *S*-adenosylmethionine dependent methyltransferases responsible for cyclopropyl
250 group modifications of mycolates and a strain additionally lacking oxygenated functional
251 groups (Barkan et al., 2012). Results of using these strains in a murine model of infection
252 show severe attenuation and changes in antigen-specific immune response dependent on the
253 presence of specific modifications. Deletion of the *hma* gene, which is essential for synthesis
254 of both oxygenated mycolate types (methoxy- and ketomycolates) shows that these forms
255 contribute to cell wall integrity, but also seem to be important for specifically inhibiting IL-
256 12p40 mediated responses (Dao et al., 2008; Dubnau et al., 2000). Considering the
257 complexity of the biosynthesis of mycolic acids and the apparent interconnectedness of the
258 enzymes in this pathway by protein-protein interactions (Cantaloube et al., 2011; Veyron-
259 Churlet et al., 2005), mutations in genes encoding core components of the fatty acid synthase
260 system II (FASII), such as *kasB* and *hadC* (Fig. 2A) seem to affect the mycolic acid
261 composition and, consequently, the virulence of respective strains (Bhatt et al., 2007; Slama et
262 al., 2016). Oxygenated mycolates, in particular, are crucial for forming so-called foamy
263 macrophages – differentiated infected macrophages rich in lipid droplets which enable the
264 persistence of the bacilli (Dkhar et al., 2014; Peyron et al., 2008) and recent steps in
265 elucidating the biosynthesis and metabolism of these mycolate classes (Madacki et al., 2018)
266 could provide new clues for studying their impact on the virulence and persistence of
267 mycobacteria.

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

274 strains has naturally raised a question of their involvement in tuberculosis pathogenesis, with
275 rather ambiguous answers when using PKS mutants *in vivo* (Rousseau et al., 2003b, 2003a).
276 A recent study analyzed multiple deletion strains deficient in DAT, PAT and SL synthesis,
277 but also in PDIM, which contain methyl branched acyl chains, with the conclusion that all of
278 the mentioned lipids contribute to phagosome maturation arrest, but with PDIM having a
279 more dominant role in this process, thus masking the effect of the former (Passemar et al.,
280 2014).

281 The role of sulfolipids in virulence has been questioned previously, notably by Rousseau et
282 al., 2003b. However, the production of SL was more recently linked to the PhoP/R two
283 component regulatory system, suggesting that PhoP-deficient strains have no or very low
284 expression of genes encoding for proteins involved in SL biosynthesis (Gonzalo-Asensio et
285 al., 2014), with possible implication on virulence. Additionally, using an approach aimed at
286 identifying factors modulating NF- κ B-dependent signaling, a transposon mutant defective in
287 SL biosynthesis was identified, showing that SL are Toll-like receptor 2 antagonists (Blanc et
288 al., 2017). Methyl-branched fatty acid-containing lipids of *M. tuberculosis* apparently have a
289 buffering role, as polyketide synthases incorporate toxic propionate, which comes from
290 cholesterol catabolism, a significant energy source for the intracellular bacilli (Lee et al.,
291 2013). Apart from their immunogenic properties, PDIM were shown to have an important
292 structural role as components of the mycomembrane, but it was also suggested that their
293 incorporation into the host plasma membrane is able to alter its biophysical properties with
294 downstream effects on phagocytic receptors (Astarie-Dequeker et al., 2009; Camacho et al.,
295 2001). Furthermore, it was shown that PDIM are indispensable for efficient phagosomal
296 rupture mediated by ESX-1, a remarkable example of coordinated usage of proteins and
297 lipids, which awaits more mechanistic insights (Augenstreich et al., 2017; Quigley et al.,
298 2017). Another group of polymethylated acyl chain containing molecules found in
299 mycobacteria are lipooligosaccharides (LOS), which are, however absent in *M. tuberculosis*.
300 Interestingly, these surface molecules are present in *Mycobacterium canettii* strains, which
301 bare the closest resemblance to the progenitor of *M. tuberculosis* (Supply et al., 2013;
302 Boritsch et al., 2016b). It was recently ascertained that LOS are responsible for the
303 characteristic smooth colony morphology of *M. canettii* strains, owing to the presence of an
304 intact dual *pks5* locus, which has apparently undergone homologous recombination resulting
305 in loss of selected *pks5* functions and *pap*-encoded acyltransferase activity in *M. tuberculosis*
306 during its evolution into a strict pathogen (Boritsch et al., 2016a). This recombination event
307 might have resulted in an evolutionary advantage for *M. tuberculosis*, judging from the

308 increased fitness and virulence of spontaneous rough morphotype and LOS-deficient *M.*
309 *canettii* K compared to its smooth counterpart. Another group of PKS-synthesized lipids,
310 phenolic glycolipids (PGL), is found only in a subset of *M. tuberculosis* strains, such as the
311 hypervirulent Beijing lineage, due to a frameshift mutation in the *pks15/1* gene (Constant et
312 al., 2002). Although immunomodulatory activity of PGLs from *M. tuberculosis* has been
313 described (Reed et al., 2004), their reported contribution to the hypervirulent phenotype of the
314 Beijing strains is not fully understood (Huet et al., 2009; Sinsimer et al., 2008).

315 The abundant mannosylated components of the cell envelope of mycobacteria –
316 phosphatidylinositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM)
317 were extensively studied for their importance in host-cell recognition – see (Vergne et al.,
318 2015) for a recent review. Variations in lipoarabinomannan structure and their impact on its
319 immunogenic properties *in vitro* have attracted much attention, notably the pathogenic
320 species-specific mannose cap motif, resulting in ManLAM, which was shown to contribute to
321 phagosome maturation arrest (Chatterjee et al., 1992; Fratti et al., 2003). An additional, and
322 unusual, methylthio-D-xylose motif was also characterized in *M. tuberculosis*, binding to one
323 mannose cap per molecule of ManLAM (Joe et al., 2006; Treumann et al., 2002), while a
324 recently discovered genetic locus responsible for its biosynthesis will enable elucidation of its
325 biological role (Angala et al., 2017). Mutant strains of *M. bovis* BCG and *M. tuberculosis*
326 producing capless LAM, however, failed to show changes in *in vivo* replication and cytokine
327 production, rather suggesting a redundant function (Afonso-Barroso et al., 2012) which
328 prompts deeper research into the biological functions of this important glycoconjugate.

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

342 2018) (Fig. 1). EspC, for example was shown to form a filamentous structure in the
343 mycobacterial cell envelope, which could theoretically be embedded in the mycomembrane
344 layer (Lou et al., 2017).

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

365

366 **4 Miscellaneous factors**

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

376 al., 2017; Senaratne et al., 2008; Shimono et al., 2003; Ekiert et al., 2017). Also, research of
377 gene and posttranslational regulation has also pointed out the importance of DosR, WhiB,
378 PhoP (Geiman et al., 2006; Smith et al., 2017), adenylyl cyclases (Samanta et al., 2017;
379 Shleeva et al., 2017), and protein kinase *pknG* (Khan et al., 2017; Rieck et al., 2017), and new
380 insights on manipulation of small molecules interfering with cytosolic surveillance are
381 emerging (Dey et al., 2015, 2017). Finally, in our selection of recently identified and/or
382 confirmed virulence factors of *M. tuberculosis* that are presented in this review, we also
383 would like to highlight a dedicated gene cluster, named *moaAI-DI*. The proteins encoded by
384 this cluster have been recently found to enable the tubercle bacillus to respire nitrate and to
385 survive oxygen depletion, a feature which seems particularly important in hypoxic
386 granulomas in the host (Levillain et al., 2017). Strikingly, this gene cluster, which is involved
387 in the biosynthesis of the molybdenum cofactor seems to have been acquired horizontally
388 together with its hypoxia-responsive transcriptional regulator by a recent common ancestor of
389 the tubercle bacilli from plasmids of environmental bacteria of another phylum (β -
390 proteobacteria *Burkholderia vietnamiensis*) (Levillain et al., 2017) (Fig. 3A). The specific
391 acquisition by tubercle bacilli is underlined by the absence of the *moaAI-DI* cluster in closely
392 related mycobacteria (*M. marinum*, *Mycobacterium kansasii*) (Fig. 3B) that have been used as
393 model organisms for studying the evolution and emergence of *M. tuberculosis* (Stinear et al.,
394 2008; Wang et al., 2015). Apart from the MTBC strains, the *moaAI-DI* genetic locus is also
395 present in *M. canettii* strains, although it is not complete in all the strains
396 (<http://genolist.pasteur.fr/CanettiiList/>), which suggests that after the acquisition of the
397 *moaAI-D* region via horizontal transfer, parts of the locus were lost in a subgroup of *M.*
398 *canettii* strains. However, in the MTBC this locus is highly conserved, suggesting that the
399 associated function in nitrate respiration (Williams et al., 2014) is important and a matter of
400 selection, apparently giving MTBC members some advantages for the survival in mammalian
401 hosts (Levillain et al., 2017).

402

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

410 2014), whereby the latter hypothesis becomes more and more accepted as it is based on the
411 analysis of ancient DNA samples and less on estimations derived from genome comparisons
412 of extant mycobacterial strains (Kay et al., 2015; Mokrousov, et al., 2017). However, it should
413 also be repeated here that the last common ancestor of the MTBC was likely a strain that
414 resembled *M. canettii*, which underwent a phenomenal clonal expansion by becoming a
415 dedicated pathogen. It is well known that the MTBC besides *M. tuberculosis* also harbors
416 very closely related relatives that infect selected other mammalian species than humans as
417 their preferential hosts. Interestingly, these animal-adapted species seem to have evolved from
418 a sublineage of the *M. tuberculosis* complex, which is characterized by the deletion of the
419 region RD9 (Brosch et al., 2002; Orgeur and Brosch, 2018), and also comprises the two *M.*
420 *africanum* lineages L5 and L6, which cause tuberculosis in humans in West Africa (Ates et
421 al., 2018a; de Jong et al., 2010; Otchere et al., 2018). As such, *M. africanum* strains seem to
422 be closely related to the common ancestor of animal strains and it will be of particular interest
423 to determine which molecular factors have been involved in the adaptation processes of these
424 tubercle bacilli to new animal hosts (Gonzalo-Asensio et al., 2014; Malone et al., 2018;
425 Orgeur and Brosch, 2018). One surprising feature in this respect is the long-time known
426 finding that *M. tuberculosis* in contrast to *M. bovis* does not cause disease in cattle, which was
427 recently re-established in an advanced cattle infection model (Villarreal-Ramos et al., 2018).
428 These are just another few examples of how complex the relationship between tubercle bacilli
429 and their various hosts are. Similar processes have certainly also contributed to the emergence
430 of *M. tuberculosis* strains from probably less virulent and much less prevalent ancestor pools
431 (resembling *M. canettii* strains) into the professional human pathogens that they represent
432 today. Without doubt, such factors make up the highly complex mosaic scaffold of
433 mycobacterial virulence, for which we have tried to highlight some of the key players that we
434 consider having an important impact on virulence. This said, there is certainly much more
435 room for studying further virulence factors of *M. tuberculosis*, as well as host immunity
436 factors that have not been covered in this review, but which can also contribute strongly to
437 advance or restrict disease progression, and thereby influence the outcome of infection. The
438 particularities of TB as disease in relation to the host and the extremely wide, and often
439 specific distribution of certain *M. tuberculosis* strain families in the global human population
440 make the infection dynamics associated to TB very complex. Research on TB-causing
441 mycobacterial pathogens will therefore need to continue for a better understanding of the
442 evolutionary adaptation processes that made *M. tuberculosis* such a powerful and devastating
443 pathogen and for finding new possible solutions to counteract this situation.

444

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446

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453

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1128 **Figure legends:**

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1130 Figure 1: Schematic representation of three types of secretion systems present in
1131 mycobacteria – the general Sec secretory pathway, the TAT pathway and the ESX / type VII
1132 secretion systems. The three-dimensional structure of ESX-5 system from *Mycobacterium*
1133 *xenopi* as determined by electron microscopy and single particle analysis (Beckham et al.,
1134 2017) is also shown.

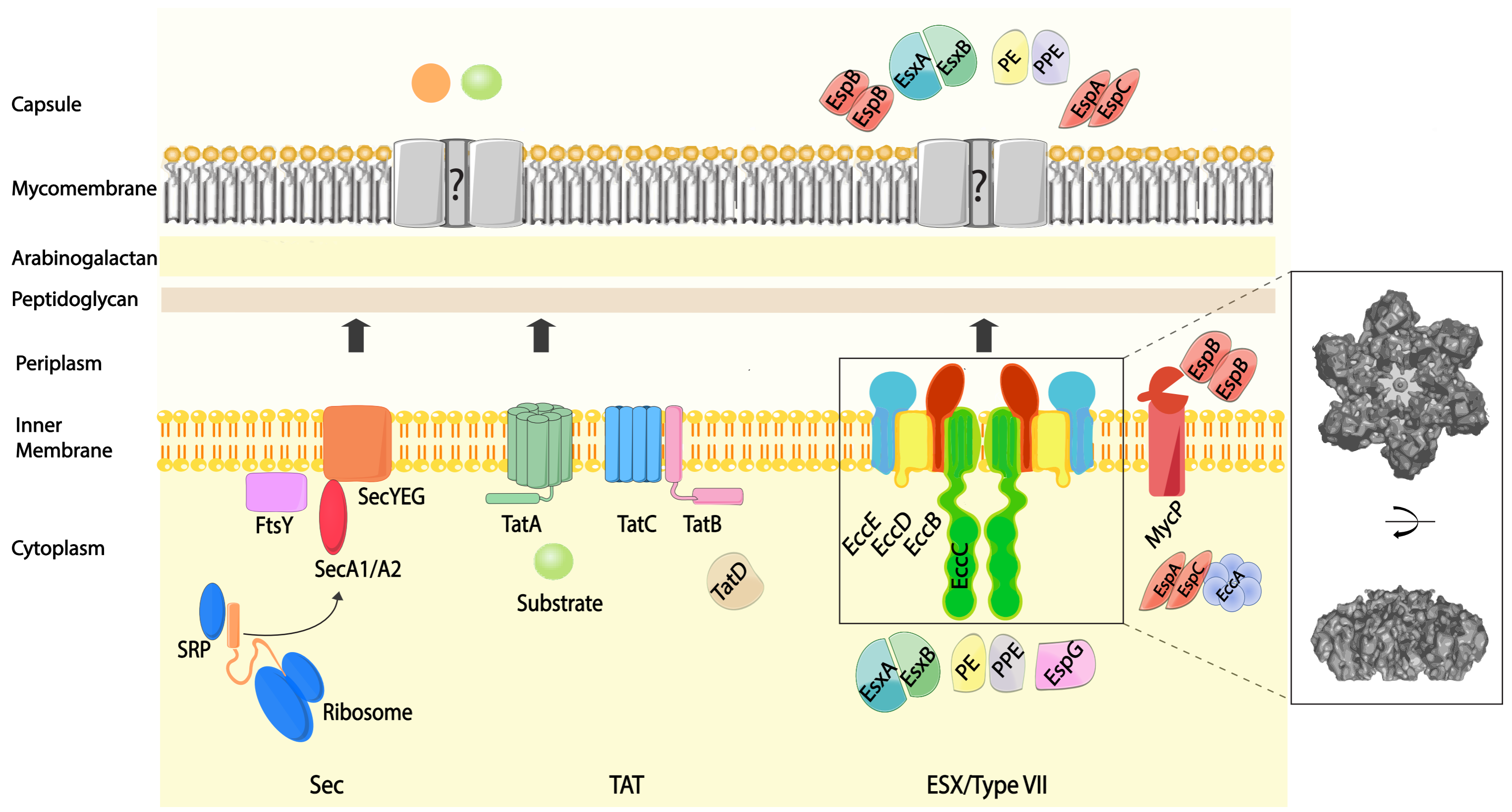
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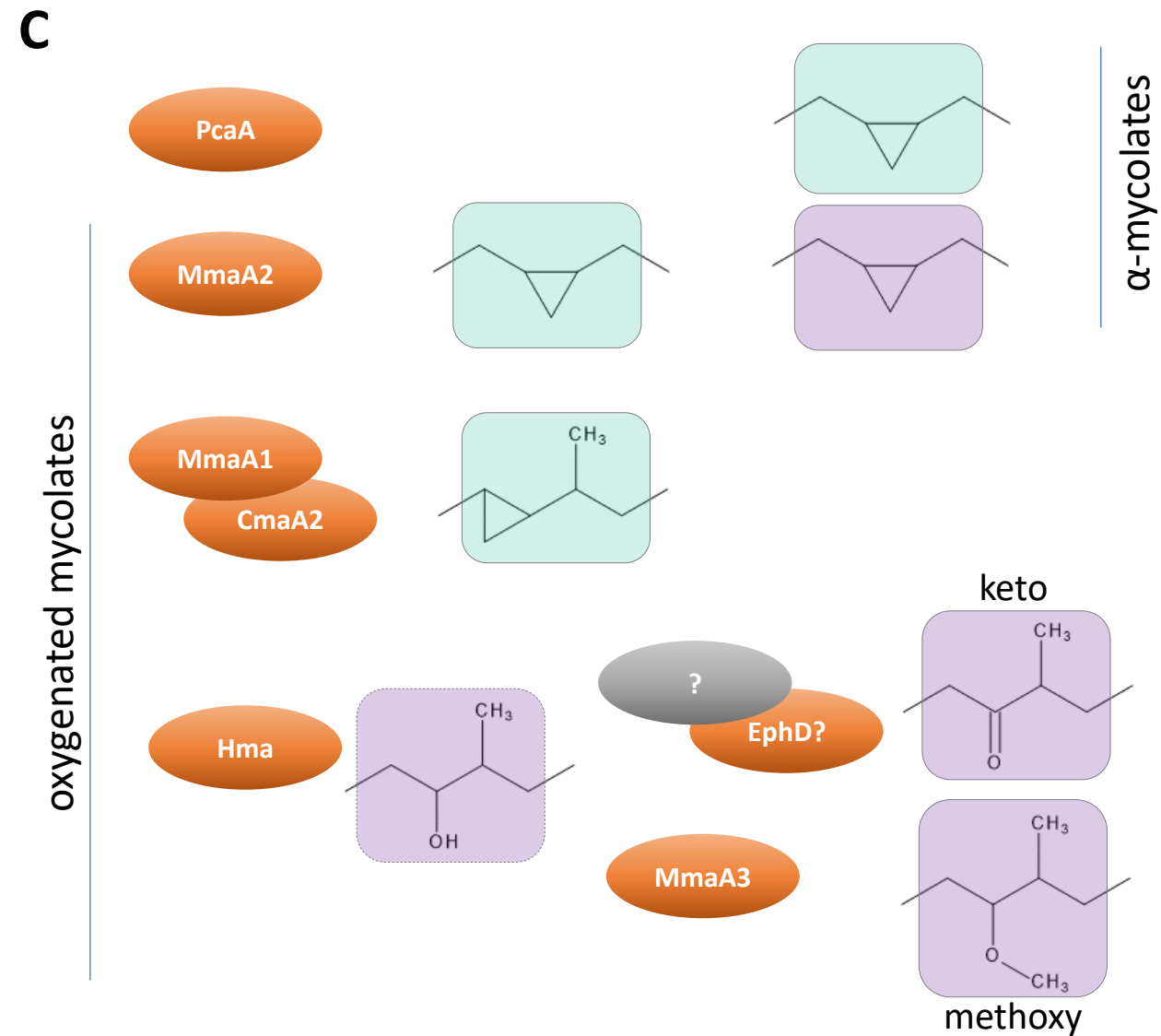
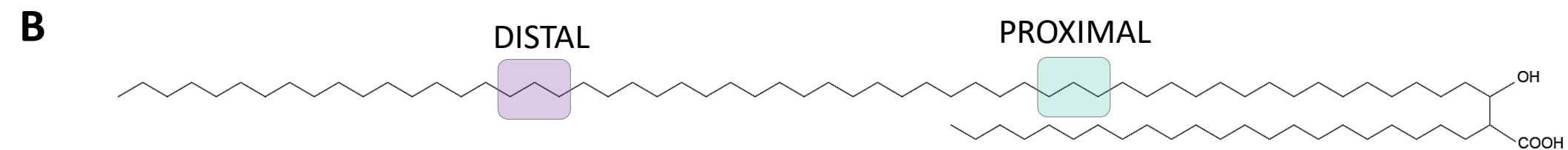
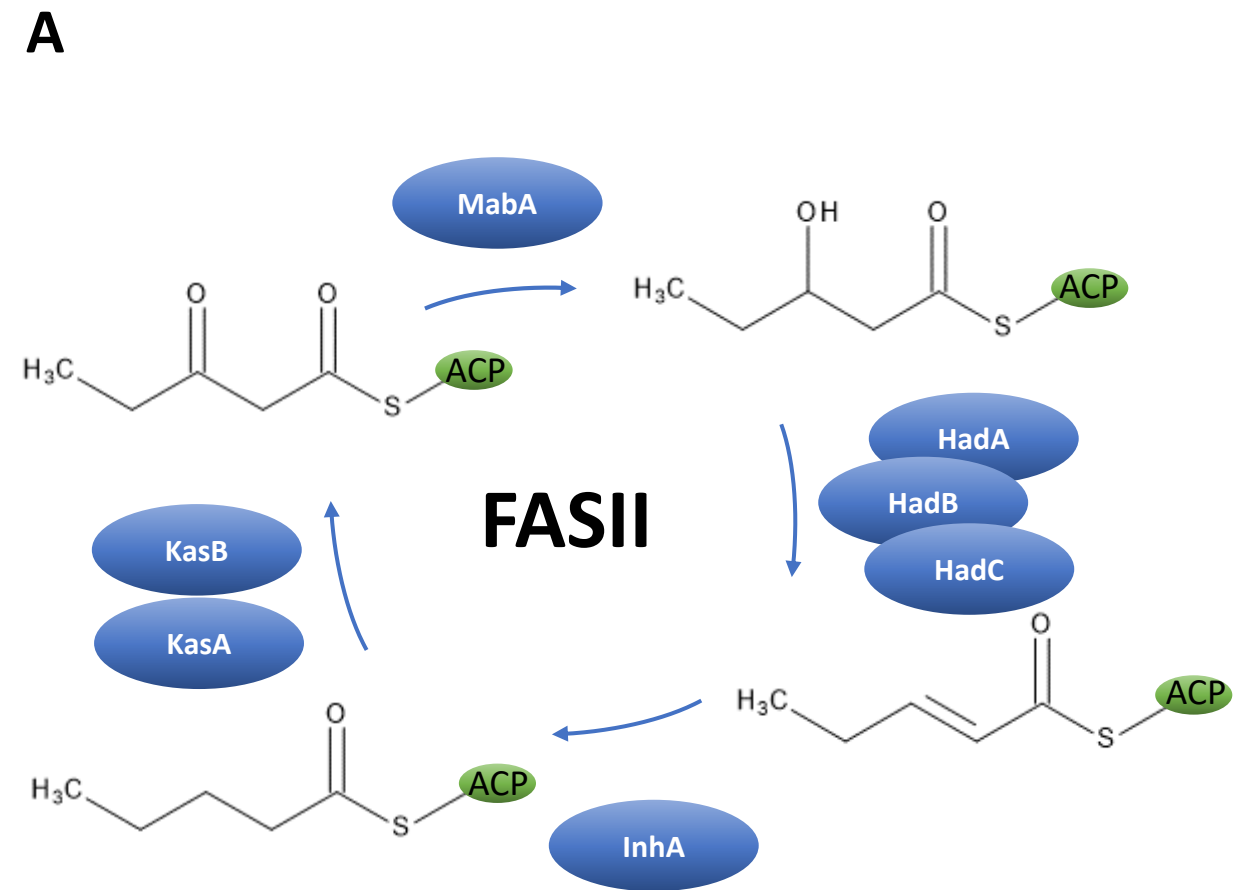
1136 Figure 2: Functional groups are attached to the main (meromycolic) hydrocarbon chain of
1137 mycolic acids likely during its elongation by fatty acid synthase system II (FASII) (A) in two
1138 distinct positions relative to the carboxyl group (B) – distal and proximal – resulting in
1139 dicyclopropylated α -mycolates and oxygenated methoxy- and ketomycolates (C). The
1140 mycolic acid modifying enzymes (in orange) are connected to the core enzymes of the FASII
1141 system (in blue) through protein-protein interactions.

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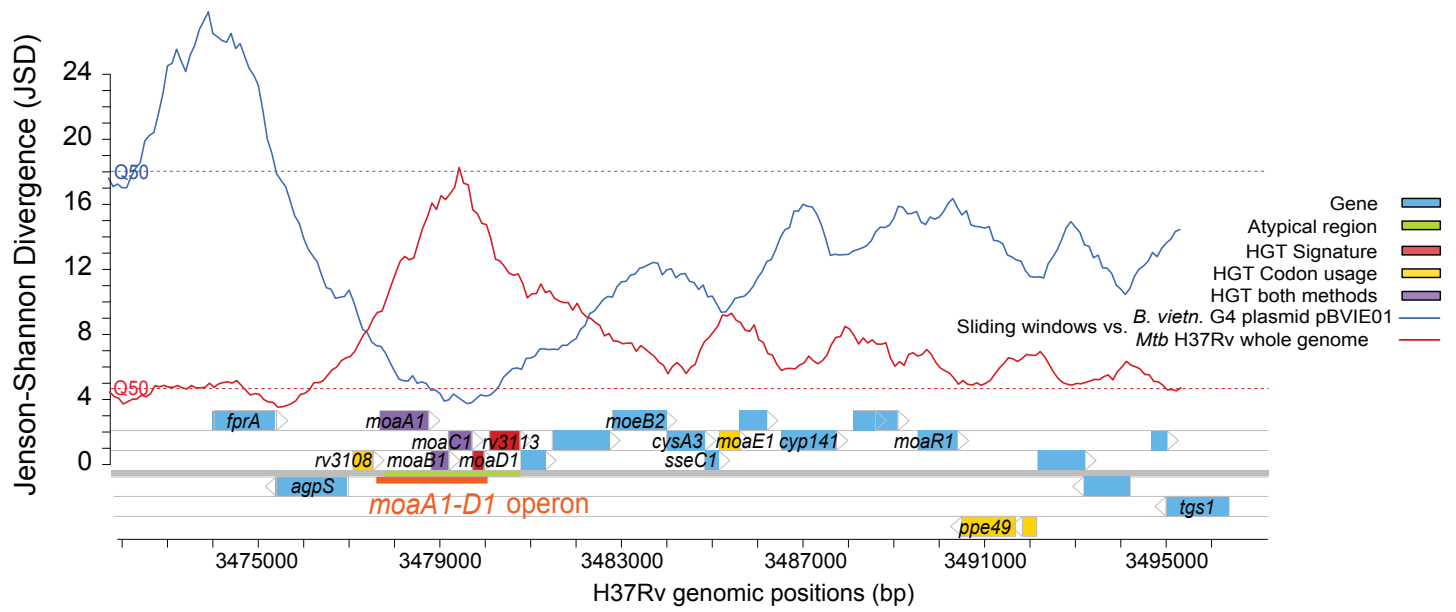
1143 Figure 3: Example of a genomic locus (*moaA1-D1* locus) acquired by tuberculosis-causing
1144 mycobacteria: (A) Jensen-Shannon divergence profiles of the *M.tuberculosis* genomic region
1145 harboring the Moco-1 island and its genomic context. The red curve represents the divergence
1146 between the signature of the whole *M. tuberculosis* H37Rv genome and that of the sliding
1147 windows (5-kb long, 100-bp step) over the region. The blue curve represents the divergence
1148 between the signature of the *Burkholderia vietnamiensis* G4 plasmid pBVIE01 and that of the
1149 sliding windows over the region, as described in detail by Levillain et al., 2017. Image
1150 adapted from Ref.(Levillain et al., 2017); (B) genomic region of the horizontally transferred
1151 *moaA1-D1* locus in *M. tuberculosis* and its flanking regions containing conserved
1152 mycobacterial genes in comparison to the orthologous regions in *Mycobacterium marinum* M
1153 and *Mycobacterium kansasii* ATCC 12478. Note that *M. marinum* M shows the minimal gene
1154 content in this genomic region, while in *M. tuberculosis* the *moaA1 -D1* gene cluster showing
1155 similarity to the *B. vietnamiensis* G4 plasmid pBVIE01 encoded genes has been inserted in
1156 addition to mycobacterial genes involved in molybdenum cofactor biosynthesis. In *M.*
1157 *kansasii* ATCC 12478, genes with different functions, including one encoding a putative
1158 polyketide synthase, are present in the genomic locus. Percentages on grey lines represent
1159 amino acid identity values between gene products of respective strains. Color coding of genes
1160 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

