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1 Review

2 *In vivo* methods to study protein-protein interactions 3 as key players in *Mycobacterium tuberculosis* 4 virulence

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10 **Abstract:** Studies on Protein-Protein interactions (PPI) can be helpful for the annotation of
11 unknown protein functions and for the understanding of cellular processes, such as specific
12 virulence mechanisms developed by bacterial pathogens. In that context, several methods have
13 been extensively used in recent years for the characterization of *Mycobacterium tuberculosis* PPI to
14 further decipher TB pathogenesis. This review aims at compiling the most striking results based on
15 *in vivo* methods (yeast and bacterial two-hybrid systems, protein complementation assays) for the
16 specific study of PPI in mycobacteria. Moreover, newly developed methods, such as in-cell native
17 mass resonance and proximity-dependent biotinylation identification, will have a deep impact on
18 future mycobacterial research, as they are able to perform dynamic (transient interactions) and
19 integrative (multiprotein complexes) analyses.

20 **Keywords:** tuberculosis; *Mycobacterium*; protein-protein interactions; virulence

21

22 1. Introduction

23 *Mycobacterium tuberculosis* (*Mtb*) is the main causative agent of human tuberculosis (TB), which
24 is the leading global cause of death due to a single infectious agent. In 2017, TB killed an estimated
25 1.6 million people, according to the World Health Organization. In addition, there is an alarming
26 increase in multi-drug resistant TB cases (0.6 million cases in 2017). Therefore, actions to fight TB
27 have to be urgently taken and understanding the mechanisms underpinning mycobacterial
28 virulence, such as signaling pathways [1], transport across the mycobacterial cell wall [2] or lipid
29 metabolism [3], may be useful to tackle TB.

30

31 Proteins perform various key roles in bacteria (enzymatic reactions, transport, DNA replication,
32 etc), either alone or in association with other partners as part of stable or dynamic complexes. Thus,
33 elucidating the role of individual proteins is essential to understand the physiology of bacteria,
34 including *Mtb*. Moreover, deciphering Protein-Protein Interactions (PPI) is crucial not only to
35 understand bacterial physiology but also to elucidate host-pathogen interactions [4]. In addition,
36 studying PPI may facilitate the discovery of unknown protein functions by the 'guilty by association'
37 principle, implying that the partner(s) of a protein with unknown function may provide valuable
38 information about the function of that protein [5]. This may potentially lead to the identification of
39 new antibacterial drug targets.

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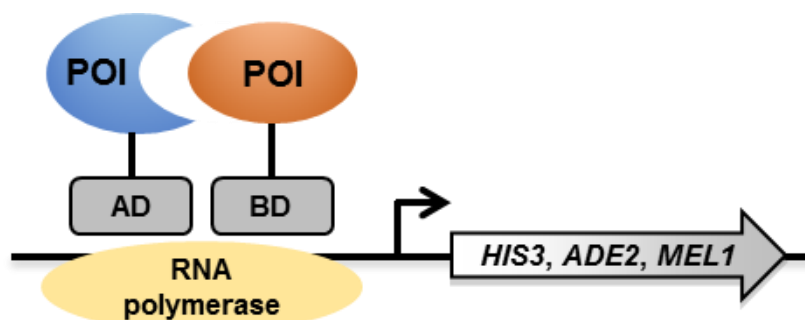
41 The aim of this review is to provide an overview of the *in vivo* methods used for the
42 characterization of PPI in mycobacteria and to highlight the pros and cons for each method. Several
43 examples will illustrate how these studies contributed to decipher the mycobacterial interactome,

44 providing worthy insights into *Mtb* virulence mechanisms [6]. This review will focus only on *in vivo*
 45 methods, and *in vitro* methods, such as co-precipitation, surface plasma resonance or isothermal
 46 titration calorimetry, will not be discussed here.

47 2. Yeast two-hybrid (Y2H) system

48 2.1. Principle

49 The Y2H system is based on the reconstitution of an active transcriptional activator (TA) in
 50 yeast (e.g. GAL4 or LexA) [7]. The proteins of interest (POIs) are produced as chimeric proteins with
 51 the DNA-binding domain (BD) or the activating domain (AD) of the TA (Figure 1). If the two
 52 proteins under investigation interact, the BD and AD are close enough to each other to allow the
 53 transcription of reporter genes, usually auxotrophic markers (*HIS3*, *ADE2* and *MEL1*) or *lacZ*, which
 54 in turn allows yeast colonies to grow on selective media or to change color on colorimetric media.



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56

Figure 1. Schematic representation of the Y2H system.

57 2.2. The Y2H system to study mycobacterial PPI

58 2.2.1. Signaling pathways

59 Sigma factors are subunits of the RNA polymerase complex required for transcriptional
 60 initiation of specific sets of genes. As rapid adaptation is key to the success for bacterial pathogens,
 61 sigma factors play a critical role in *Mtb* physiology and virulence [8]. Among the dozen of sigma
 62 factors in *Mtb*, SigA, also called RpoV, is essential for growth and is involved in the transcription of
 63 housekeeping genes [9]. To study mycobacterial PPI involving SigA, the Y2H system was used to
 64 screen a *Mtb* H37Rv library, which led to the identification of the transcriptional regulator WhiB3 as
 65 an interactor of SigA/RpoV [10]. In addition, a single amino acid change in SigA/RpoV (R515H) was
 66 sufficient to abolish its interaction with WhiB3 in the Y2H system [10]. Another transcriptional
 67 regulator, WhiB1, was shown to interact with the alpha-glucan branching enzyme GlgB [11]. SigF is
 68 the general stress response sigma factor of *Mtb* and is responsible for the regulation of genes
 69 involved in cell wall protein synthesis and in the survival of the bacilli in the host [12,13]. The Y2H
 70 system was also used to study the interactions between anti-anti-sigma factor, anti-sigma factor
 71 RsbW and sigma factor SigF [14].

72 Besides sigma factors, the *Mtb* genome encodes a dozen two-component systems (TCS),
 73 allowing gene expression to adapt in response to a wide variety of signals. Some of these TCS were
 74 shown to be involved in the regulation of virulence [15]. The Kdp signal transduction pathway
 75 appears to be the primary response mechanism to osmotic stress, which is mediated by differences
 76 in the potassium concentrations within the bacteria. The N-terminal sensing module of the histidine
 77 kinase KdpD interacts with a portion thought to be cytosolic of two membrane lipoproteins, LprF
 78 and LprJ, to modulate *kdp* expression [16]. Another study assessed pairwise interactions in the Y2H
 79 system between histidine kinases and response regulators of all the mycobacterial TCS in order to
 80 assess crosstalks between the different TCS [17].

81 The *Mtb* genome also encodes eleven Serine/Threonine Protein Kinases (STPK), from PknA to
82 PknL. As the *Mtb* phosphoproteome includes hundreds of Ser- and Thr-phosphorylated proteins
83 that participate in many aspects of *Mtb* biology (signal transduction, cell wall synthesis,
84 pathogenesis, etc), STPK are critical for the regulation of *Mtb* physiology [18]. In an extensive study,
85 492 STPK interactants were identified by a *Mtb* proteome microarray [19]. To confirm the *in vitro*
86 screening, the Y2H system was further used to assess the interactions between 75 randomly-selected
87 interactants with PknB, PknD, PknG and PknH. However, only 52% (39 out of 75) of the STPK
88 interactants could be confirmed by the Y2H system, which may be due to the fact that PknB, PknD
89 and PknH were tested without their membrane domain, as this could have been detrimental in the
90 Y2H system [19].

91 2.2.2. *Mtb* cell division

92 As for other living organisms, mycobacterial cell growth and division needs to be tightly
93 organized and regulated [20]. In particular, divisome assembly depends on the proper localization
94 of FtsZ in order to form the Z ring structure [20]. Thus, a Y2H screening was performed using *Mtb*
95 FtsZ as a bait, which led to the identification of SepF (Rv2147c), an essential protein of the division
96 machinery in mycobacteria [21].

97 Reactivation of dormant *Mtb* requires the resuscitation-promoting factors (Rpf), which are
98 peptidoglycan-hydrolyzing enzymes [22]. The Y2H system was used to identify a RpfB and RpfE
99 interactant, named RipA for Rpf-interacting protein A [23]. Additional work performed with RipA
100 as a bait in the Y2H system further identified the protein PBP1/PonA1 as a new partner, potentially
101 modulating the RipA-RpfB cell wall degradation activity [24].

102 2.2.3. *Mtb* cell wall composition

103 Mycolic acids are essential lipid components of the mycolic
104 acid-arabinogalactan-peptidoglycan complex (MAPc) in the *Mtb* cell wall and they contribute
105 directly to the pathogenicity of *Mtb* [25]. The Y2H system was extensively used to demonstrate that
106 the discrete enzymes of the Fatty Acid Synthase-II (FAS-II) system interact with each other during
107 mycolic acid biosynthesis, suggesting the existence of specialized and interconnected protein
108 complexes [26-28].

109 Another study using the Y2H system showed that Rv2623, a universal stress-response protein,
110 and Rv1747, a putative ABC transporter, interact with each other to regulate mycobacterial growth
111 by potentially impeding Rv1747 function as a phosphatidylinositol mannoside (PIM) transporter
112 [29]. PIM are immunologically active lipids that can modulate the host immune response [30,31].

113 2.2.4. Secretion of *Mtb* virulence factors

114 ESAT-6 and CFP-10 are both secreted antigens, which play a key role in *Mtb* virulence [32,33].
115 The Y2H system helped to demonstrate that EccCa1, EccCb1 and EccD1, which are components of
116 the type VII secretion system ESX-1 [34,35], are required for ESAT-6/CFP-10 secretion [36]. In
117 addition, a single amino acid change in the C-terminal region of CFP-10 was enough to abolish the
118 CFP-10/EccCb1 interaction in the Y2H system, and to prevent secretion of the ESAT-6/CFP-10
119 complex [37]. Similarly to CFP-10, the C-terminal region of EspC, another ESX-1 substrate, was
120 shown to interact with Rv3868, a cytosolic ATPase, by a Y2H approach [38].

121 2.2.5. Regulation of mycobacterial protease activity

122 Mycobacterial proteases play critical roles in pathogenesis [39]. For instance, the site-2 protease
123 Rip1 (Rv2869c) is a major virulence determinant in *Mtb* [40,41]. A Y2H screening performed using
124 the Rip1 PDZ domain against a *Mtb* library led to identification of PDZ-interacting protease

125 regulators 1 and 2 (Ppr1 and Ppr2, corresponding to Rv3333c and Rv3439c, respectively) and these
 126 interactions are thought to prevent nonspecific activation of the Rip1 pathway [42].

127 2.3. Pros and cons

128 The Y2H system allows direct assessment of pairwise interactions between partners in an *in vivo*
 129 context. However, as the readout is based on transcription factors active in the nucleus of the yeast
 130 cell, the Y2H system requires nuclear translocation of the proteins under study. Thus,
 131 membrane-associated proteins are difficult if not impossible to study in this system (Table 1).
 132 Furthermore, only two (or three in the case of a Y3H system) partners can be studied at a time. In
 133 addition, the Y2H system is not suitable for the study of PPI in their natural cellular context, and
 134 specific mycobacterial Post-Translational Modifications (PTM) or cofactors may be lacking in yeast
 135 (Table 1).

136 **Table 1.** Comparison of the different techniques used to study mycobacterial PPI.

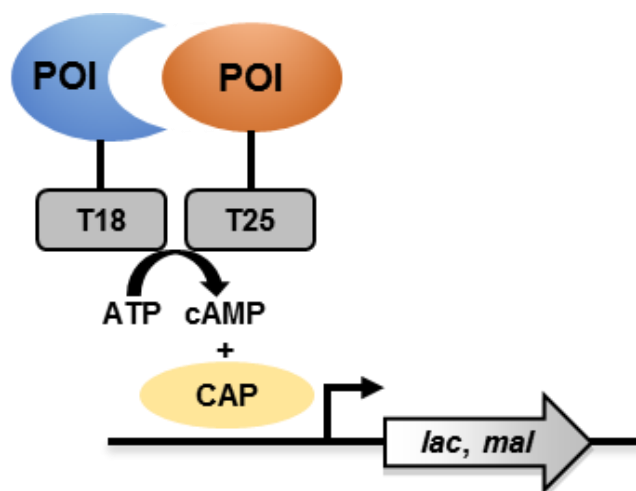
Methods	Contact	Membrane proteins	Nature of the interaction	Cellular context	PTM and cofactors	HTS ¹
Y2H	direct	no	binary	no	no	yes
BACTH	direct	yes	binary	yes/no	yes/no	yes
M-PFC	direct	yes	binary	yes	yes	yes
Split-Trp	direct	yes	binary	yes	yes	yes
Crosslinking	direct	yes	complex	yes	yes	no
In-cell NMR	proximity	yes	complex	yes/no	yes/no	no
Biotinylation	proximity	yes	complex	yes	yes	yes

137 ¹ HTS, High-Throughput Screening

138 3. Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) system

139 3.1. Principle

140 The BACTH system is based on the interaction-mediated reconstitution of an active *Bordetella*
 141 *pertussis* adenylate cyclase (CyaA) in *Escherichia coli* [43-45]. POIs are genetically fused to the
 142 N-terminal or C-terminal ends of the subunits T18 or T25 of CyaA (Figure 2). The enzyme is inactive
 143 when T18 and T25 are physically separated. When the POIs interact, the proximity of T18 and T25
 144 allows the generation of cyclic AMP (cAMP), which then binds to the Catabolite Activator Protein
 145 (CAP). This cAMP/CAP complex then activates the transcription of reporter genes (*lac* and *mal*
 146 operons). As *lac* and *mal* operons are involved in lactose and maltose catabolism, respectively, this
 147 allows *E. coli* to grow on media on which lactose or maltose is the unique carbon source.



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Figure 2. Schematic representation of the BACTH system.

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3.2. The BACTH system to study mycobacterial PPI

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3.2.1. Signaling pathways

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Mtb SigE plays an important role in the intracellular life of mycobacteria and regulates the expression of several genes that are important for maintaining the integrity of the cell envelope during stress, particularly during macrophage infection, since SigE is required to arrest phagosome maturation [46,47]. SigE interacts with the anti-sigma factor RseA in the BACTH system, and using this system residues C70 and C73 of RseA have been shown to be required for full interaction, which prevents the transcription of genes that are controlled by SigE [48].

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The BACTH system was also used to study the interactions between components of TCS, such as the C-terminal domain of the response regulator MtrA and the histidine kinase MtrB [49]. Although the environmental signals sensed by MtrA/B are unknown, this TCS is essential for mycobacterial growth. On the other hand, overexpression of *mtrA* was shown to impede *in vivo* proliferation of *Mtb* [50,51]. In another study, the sensor kinase KdpD was found to interact in the BACTH system with the membrane peptide KdpF, potentially altering KdpABC transporter function [52]. In the same study, a screening was performed using KdpF as a bait against a *Mtb* H37Rv DNA library, which led to the identification of MmpL7 and MmpL10 as interactors [52]. These proteins are members of the MmpL protein family involved in lipid and iron transport in mycobacteria [53,54]. It was further shown that KdpF also interacts with the nitrosative stress detoxification proteins NarI and NarK2, as well as with a protein highly induced upon nitrosative stress, Rv2617c [55]. This PPI network suggests that the KdpF peptide could promote the degradation of these partners involved in nitrosative stress, leading to decreased intracellular multiplication of the mycobacteria [55].

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3.2.2. Cell division

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The BACTH system was also used to characterize the mycobacterial cell division. It allowed the identification of interactions between FtsW, FtsZ and PbpB [56]. Another study demonstrated that FtsZ is able to interact with ClpX, the substrate-recognition domain of the ClpXP protease, potentially modulating Z-ring structure formation and negatively regulating FtsZ polymerization [57]. FtsZ also interacts with CrgA (Rv0011c), a protein that possibly facilitates septum formation [58]. Another study showed that the membrane protein CwsA (Rv0008c) interacts with CrgA and Wag31, both involved in mycobacterial peptidoglycan biosynthesis [59]. Together these studies highlight the value of the BACTH system to characterize the mycobacterial divisome [60].

181

3.2.3. *Mtb* cell wall composition

182 A BACTH screening using as a bait KasA, a component of FAS-II system, revealed that KasA
183 interacts with PpsB and PpsD, which are two enzymes involved in the biosynthesis of lipid
184 phthiocerol dimycoserolate (PDIM). This suggests a possible transfer of lipids between the FAS-II
185 system and the PDIM biosynthetic pathways [61], highlighting the importance of PPI in the course of
186 mycobacterial cell wall biosynthesis. Similar to mycolic acids, PDIM are involved in mycobacterial
187 virulence [62,63].

188 EccA1 is an ATPase and belongs to ESX-I, the mycobacterial type VII secretion system [34]. It
189 was shown that the *Mycobacterium marinum* EccA1 activity is required for optimal mycolic acid
190 biosynthesis, probably through its interaction with FAS-II components (KasA and KasB), the
191 mycolic acid condensase Pks13 and potentially with the mycolic acid methyltransferase MmaA4
192 [64]. In addition, EchA6, a putative enoyl-CoA hydratase, also interacts with several members of the
193 FAS-II system (KasA and InhA), suggesting a possible role in feeding FAS-II with long-chain fatty
194 acids [65].

195 The BACTH system was also used to detect interactions between the transporter-like Rv3789
196 and the galactosyltransferase GltI1, involved in arabinogalactan biosynthesis, another component of
197 the mycobacterial MAPc [66].

198 Recently, a *Mtb* genome-wide screening using MmpL3 as a bait in BACTH system identified
199 several interactants related to mycolic acid biosynthesis (MmpL11 and Rv0228=TmaT), PG
200 biosynthesis (Rv3909, Rv3910 and Rv1337), glycolipid biosynthesis (Rv0227c, Rv0236c=AftD and
201 Rv1457c) and cell division (CrgA) [67].

202 3.2.4. *Mtb* virulence factors

203 The BACTH system was used to search for partners of the virulence-associated factor Erp,
204 which is required for optimal multiplication of *Mtb* in murine bone marrow-derived macrophages
205 and *in vivo* in mice [68]. This led to the identification of two putative membrane proteins, Rv1417 and
206 Rv2617c [69], the functions of which remain yet to be established.

207 MgtC is a virulence factor that participates to the adaptation of mycobacteria to magnesium
208 deprivation [70]. The BACTH system was used to assess the interactions between MgtC from *Mtb*
209 and a MgtR peptide from *Salmonella typhimurium* [71], known to promote MgtC degradation in
210 *Salmonella* [72]. Thus, the BACTH system is also useful to evaluate the anti-virulence activity of
211 peptides (or proteins).

212 HbhA is a surface-exposed adhesin that is involved in the binding of mycobacteria to
213 non-phagocytic cells, a necessary process for *Mtb* dissemination [73], and in the formation of
214 intracellular lipid inclusions [74]. The BACTH system was used to demonstrate that HbhA interacts
215 with Rv0613c and MmpL14 [75]. In addition, deletion of the orthologous gene of *rv0613c* in
216 *Mycobacterium smegmatis* prevents cell-surface exposure of HbhA [75], illustrating that the BACTH
217 system can be helpful to start deciphering novel protein secretion mechanisms.

218 A three-hybrid system was developed in *E. coli* and helped to confirm the interactions between
219 ESAT-6, CFP-10 and EccCb1 [76], as previously described with individual binary interactions
220 identified in the Y2H system [36,37].

221 3.2.5. High-Throughput screening applied to BACTH

222 The BACTH system has mostly been used to study pairwise interactions between a limited
223 number of proteins. However, a global *Mtb* PPI network was also studied using the BACTH system.
224 By using the nearly complete *Mtb* gene sets, it led to the identification of more than 8,000 interactions

225 involving 2,907 mycobacterial proteins [77]. All these potential interactions now require further
 226 validation and characterization using complementary approaches.

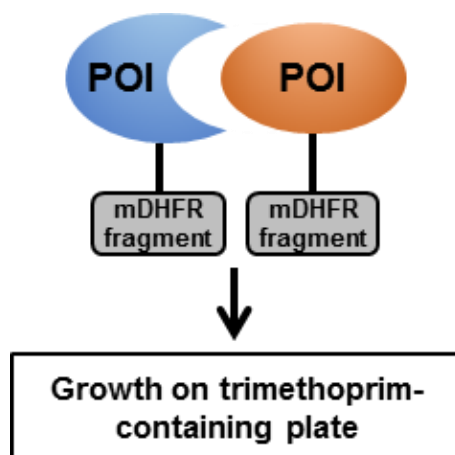
227 3.3. Pros and cons

228 Like the Y2H system, the BACTH system also permits to test direct interactions of pairwise
 229 partners in an *in vivo* environment and is limited to detect binary interactions (or potentially ternary
 230 interactions in the case of the bacterial three-hybrid system). However, unlike the Y2H system,
 231 membrane-associated proteins can be studied in the BACTH system, as long as T18 and T25 reside in
 232 the cytoplasmic compartment of the bacteria. The bacterial cellular context is partially maintained
 233 but it lacks the specificity of the mycobacterial cell wall organization (Table 1). Finally, some
 234 bacterial PTM and cofactors may be present in *E. coli*, however, all specific mycobacterial PTM and
 235 cofactors are absent (Table 1).

236 4. Methods developed for use with live mycobacteria

237 4.1. The mycobacterial protein fragment complementation (M-PFC)

238 The Y2H and BACTH systems have their limitations, as the identified interactions do not
 239 necessarily occur in their natural environment. In addition, neither system can take care of the
 240 specific mycobacterial cell wall organization, and some of the specific PTM and cofactors (Table 1).
 241 Hence, systems to directly assess PPI in a mycobacterial environment have been developed. The
 242 mycobacterial protein fragment complementation (M-PFC) technology relies on the functional
 243 reconstitution of a murine dihydrofolate reductase (mDHFR) in *M. smegmatis* [78]. The POIs are
 244 fused to complementary fragments of mDHFR (Figure 3). If the POIs interact, the reconstitution of
 245 an active mDHFR confers resistance to the antibiotic trimethoprim. This system was validated by
 246 confirming the interactions between ESAT-6 and CFP-10, membrane-associated DosS and cytosolic
 247 DosR, and membrane-associated KdpD and cytosolic KdpE [78]. The authors performed a screen
 248 using a *Mtb* library and CFP-10 as a bait, which confirmed interactions of CFP-10 with ESAT-6 and
 249 identified new interactions of CFP-10 with Rv0686, FtsQ, ClpC1, Pks13 and Rv2240c [78].
 250 Interestingly, the interaction between CFP-10 and mycolic acid condensase Pks13 could not be
 251 reproduced in the Y2H system, inferring that this interaction requires a specific mycobacterial
 252 environment to be detected [78].



253

254

Figure 3. Schematic representation of the M-PFC technology.

255 4.1.1. Signaling pathways

256 M-PFC was also used to demonstrate interactions between PknH and the response regulator
 257 DosR, demonstrating convergence between STPK and TCS signaling in *Mtb* [79]. In combination
 258 with *Mtb* proteome microarrays and Y2H approaches, M-PFC was used to further validate
 259 interactions between STPK protein interactants and the two selected STPK PknB and PknD [19].

260 4.1.2. Cell division

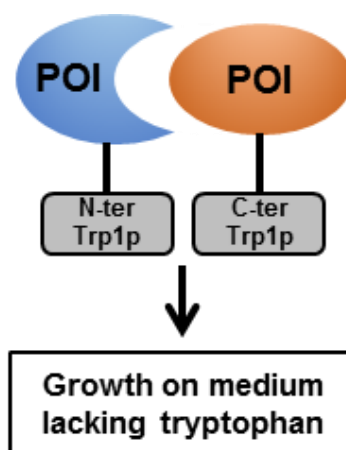
261 In agreement with the BACTH system, M-PFC confirmed interactions between ClpX and FtsZ
 262 [57]. M-PFC also confirmed interactions between FtsZ and SepF [80], independently of the screening
 263 performed in the Y2H system using FtsZ as a bait, as mentioned above [21].

264 4.1.3. Peptidoglycan biosynthesis

265 Mur synthases (MurC-F), which are essential and involved in peptidoglycan biosynthesis in
 266 mycobacteria [81], interact with regulatory proteins and proteins involved in cell division, such as
 267 PknA and PknB [82].

268 4.2. The split-protein sensor (*Split-Trp*)

269 Split-Trp (or protein fragment complementation assay) requires a tryptophan biosynthetic
 270 pathway, which is present in mycobacteria. It relies on the reconstitution of an active Trp1p enzyme,
 271 only if the POIs interact with each other (Figure 4 and Table 1). This will then allow the tryptophan
 272 auxotrophic strain of *M. smegmatis* $\Delta hisA$ to grow on media without tryptophan [83]. The validity of
 273 Split-Trp was assessed by confirming interactions between ESAT-6 and CFP-10, and the
 274 homodimerization of GlfT1 and RegX3 [83]. In parallel with M-PFC, Split-Trp was used to evaluate
 275 interactions between PknH and DosR. However, only the phosphorylation-defective form of DosR
 276 (T198A/T205A) was able to interact with PknH in this system, suggesting that Split-Trp is less
 277 sensitive than M-PFC [79].

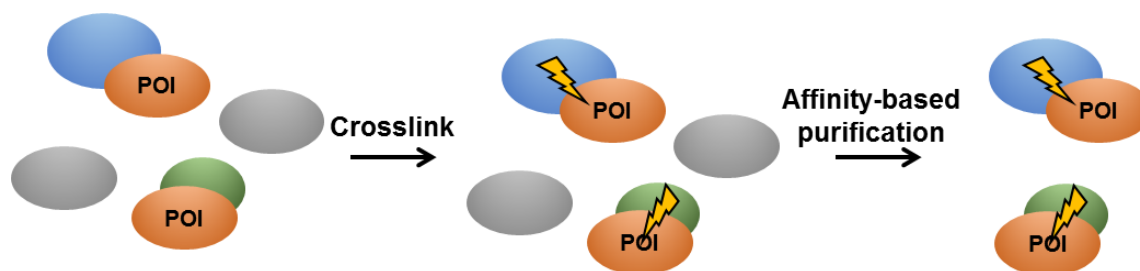


278

279 **Figure 4.** Schematic representation of the Split-Trp technology.

280 4.3. *In vivo* crosslinking in live mycobacteria

281 *In vivo* crosslinking was developed to directly address PPI in a natural environment in order to
 282 limit false positive interactions or miss transient interactions (Table 1). It relies on the use of
 283 crosslinking agents, such as formaldehyde or (sulfo-)disuccinimidyl suberate, generating covalent
 284 adducts of two spatially close proteins (Figure 5). Using formaldehyde as a crosslinking agent, *Mtb*
 285 subunit E1 of the pyruvate dehydrogenase complex was shown to interact with nine *M. smegmatis*
 286 proteins [84]. Nonetheless, this approach could generate false positives, as naturally biotinylated
 287 mycobacterial proteins may interfere with the purification protocol [84].



288

289

Figure 5. Schematic representation of *in vivo* crosslinking.

290 A more recent approach consists in incorporating the UV-crosslinking unnatural amino acid
 291 *p*-benzoylphenylalanine, added to the culture medium, via nonsense suppression in the sequence of
 292 the protein under study [85]. Upon UV irradiation of live cells, this allows the formation of a
 293 covalent adduct between the studied protein and any interactant, thus capturing physiological
 294 interactions in a native environment. This method was applied to the lipoprotein LprG [86], which is
 295 involved in cell surface exposure of lipoarabinomannan, the regulation of triacylglycerol levels,
 296 phagolysosomal fusion and *Mtb* virulence [87-89]. Among 23 identified interactants, the authors
 297 focused on the site-specific interactions of LprG with LppI and LppK, as well as on the physical and
 298 functional interactions between LprG and the mycolyltransferase Ag85A conditioning cell growth
 299 and mycolic acid composition [86].

300 4.4. Pros and cons

301 Methods developed for use with live mycobacteria are devoted to test direct interactions
 302 between potential partners within the mycobacterial environment, in the presence of an adequate
 303 cellular organization and the potentially required cofactors or PTM. M-PFC and Split-Trp can be
 304 used to characterize pairwise interactions, whereas *in vivo* crosslinking may be useful to demonstrate
 305 the existence of protein complexes (Table 1). However, this latter technique is hardly amenable for
 306 the development of a high-throughput screening system (Table 1). As distance and orientation
 307 between the tested proteins are important, Split-Trp may lead to false positive or false negative
 308 results, as shown for some ESAT-6 and CFP-10 interactions [83]. Thus, the use of several
 309 independent methods in mycobacteria is important in order to eliminate false positive or false
 310 negative results.

311 5. Conclusion and perspectives

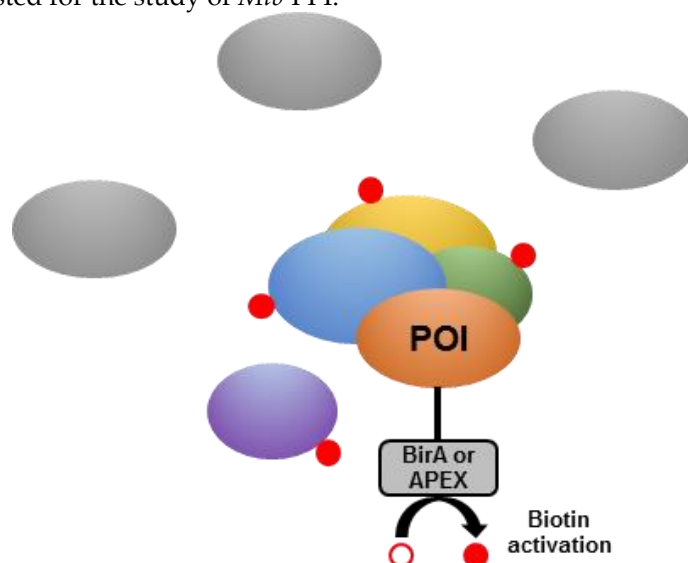
312 All the methods listed above greatly contributed to the understanding of *Mtb* virulence
 313 mechanisms by focusing on PPI. However, despite the tremendous amount of data generated by
 314 these different technologies, deciphering mycobacterial PPI in terms of multiprotein and dynamic
 315 complexes requires more specific and more appropriate systems. In that regard, novel methods,
 316 such as in-cell Native Mass Resonance (NMR) spectroscopy or the proximity-dependent
 317 biotinylation assay, appear to be very promising (Table 1).

318

319 In-cell NMR is useful to study the conformation and the dynamics of biological macromolecules
 320 (such as protein complexes) under physiological conditions (i.e. within living cells) [90]. For
 321 instance, in-cell NMR was used to study the intrinsically disordered mycobacterial protein Pup, a
 322 functional analog of ubiquitin [91]. Pup targets mycobacterial proteins for proteasome-mediated
 323 degradation, a process that is directly involved in *Mtb* virulence [92]. Pup was studied for its
 324 interaction in *E. coli* with the mycobacterial proteasomal ATPase Mpa and with the intact
 325 mycobacterial proteasome (Mpa plus *Mtb* proteasome core particle), showing that the proteasome
 326 complex had a higher affinity for Pup than Mpa alone [93]. However, the application of in-cell NMR
 327 directly in living mycobacteria remains to be tested and further developed.

328

329 Proximity-dependent biotinylation assays [94] consist in generating a hybrid protein between
 330 the POI and a biotin ligase (e.g. a variant of *E. coli* BirA [95] or *A. aeolicus* biotin ligase [96]) or an
 331 engineered ascorbic acid peroxidase (e.g. APEX [97,98]) (Figure 6). APEX catalyzes the conversion of
 332 its substrate biotin-phenol into short-lived and highly reactive radicals, leading to the covalent
 333 attachment of biotin to electron-rich amino acids (such as tyrosines) of proximal proteins [94]. As the
 334 technique is directly performed in the organism of interest, whose subcellular structures are kept
 335 intact, it greatly minimizes false-positive identifications. The hybrid protein can properly localize,
 336 perform its function and add a biotin residue to all potential partners in spatial proximity (in a 10- to
 337 20-nm radius). Once the biotin is covalently bound to the proximal proteins, classical lysis methods
 338 are not expected to interfere in the process, in contrast to other approaches, such as co-precipitation
 339 or tandem affinity purification. The bacterial lysate can then be subjected to purification using
 340 streptavidin-based beads or columns. After stringent washes, elution and tryptic digestion, the
 341 samples can be subjected to mass spectrometry analysis to detect which biotinylated proteins are
 342 enriched in the samples. This method may be particularly suitable for the study of PPI in a natural
 343 context, for particular subcellular structures or for proteins involved in specific mycobacterial
 344 processes (such as cell wall biosynthesis or virulence mechanisms). Although *Mtb* possesses a biotin
 345 synthesis pathway [99] that could interfere with this technique, the use of relevant controls (e. g. a
 346 similar production of the POI not fused to the biotin ligase) would allow the identification of a
 347 specific subset of enriched biotinylated proteins, representing either direct interactants or
 348 spatially-close partners. This technology has not yet been applied to mycobacteria, but may be
 349 worthwhile to be tested for the study of *Mtb* PPI.



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351

Figure 6. Schematic representation of the proximity-dependent biotinylation assay.

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