Title: N-acetyl-transferases required for iron uptake and aminoglycoside resistance promote virulence lipid production in *M. marinum*

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Classification:

Abstract

Mycobacterial pathogens, including *M. tuberculosis,* the cause of human tuberculosis, and the non-tubercular species, *M. marinum* lyse the host phagosome during infection. Acetylation is a conserved protein modification mediated enzymatically by N-acetyltransferases (NATs). NATs acetylate protein N^{α}-termini, lysine residues, and small molecules like antibiotics to alter bacterial physiology and pathogenesis. To define conserved mycobacterial NATs required for lytic activity, we generated and characterized *M. marinum* strains with deletions in predicted conserved NAT genes. *M. marinum* lyses red blood cells in a contact dependent manner, and screened for hemolytic activity. We found that nearly all of the conserved lysine acetyltransferases (KATs) contributed to the hemolytic activity of *M. marinum*. Hemolysis is mediated by both the ESX-1 secretion system and by phthiocerol dimycocerosate (PDIM), an outer lipid required for virulence. For several strains, the hemolytic activity of several KAT deletion strains was restored by the addition of second copy of the ESX-1 locus. Using thin-layer chromatography (TLC), we found that MbtK was essential for PDIM and phenolic glycolipid (PGL) production. MbtK is a conserved KAT required for mycobactin siderophore synthesis and virulence. We show that the addition of Mycobactin J exogenously

complements PDIM/PGL production in the *ΔmbtK* strain, linking production of these lipids to iron uptake. The Δ*mbtK M. marinum* strain was attenuated in macrophage and *Galleria mellonella* infection models. Constitutive expression of either *eis* or *papA5,* which encode a known KAT and a PDIM/PGL biosynthetic enzyme, rescued PDIM/PGL production and virulence of the Δ*mbtK* strain. Eis is an N-acetyltransferase linked to aminoglycoside resistance. We found that Eis N-terminally acetylated PapA5 *in vitro*, suggesting that Eis modification of PapA5 in the absence of MbtK restored lipid production. Overall, our study defines new connections between the MbtK and Eis NATs, and between iron uptake and PDIM and PGL synthesis in *M. marinum*. Moreover, our findings underscore the multifunctional nature of mycobacterial NATs and their connection to key virulence pathways.

Significance Statement: Acetylation is a ubiquitous modification of protein N-termini, lysine residues, antibiotics and lipids. Many of the enzymes that promote acetylation belong to the GNAT family of proteins. *M. tuberculosis* is the causative agent of Tuberculosis. *M. marinum* is a related pathogen that is well established as a model to understand aspects of how *M. tuberculosis* causes disease. In this study we sought to identify conserved GNAT proteins required for early stages of mycobacterial infection. Using *M. marinum,* we determined that several GNAT proteins are required for the lytic activity of *M. marinum.* We uncovered previously unknown connections between acetyl-transferases required for iron uptake and antimicrobial resistance, and the production of the unique mycobacterial lipids, PDIM and PGL. Importantly, our data support that acetyl-transferases from the GNAT family are interconnected, and have activities beyond those previously reported in the literature.

Introduction:

Acetylation is the movement of an acetyl group from an acetyl donor to an amine including N^{α}-amino groups (N-terminal acetylation), the ε-amine of lysine (N^{ε}-acetylation) or primary amines of nucleotides, peptides, and small molecules (1). Acetylation can be facilitated enzymatically by

53 N-acetyltransferases (NATs). N-terminal protein acetylation is considered irreversible because N-
54 terminal deacetylases have not been identified. Lysine acetylation is reversible by lysine deacety-
55 lases (2-4) . Protein acetylation regulates protein folding, stability, localization, and protein-protein
56 interactions (5-11).

57 *Mycobacterium tuberculosis* is the cause of the human disease tuberculosis (12). In 2022,
58 *M. tuberculosis* infected 10.6 million individuals, resulting in an estimated 1.3 million deaths world-
59 wide (13). *M. marinum* causes a tuberculosis-like disease in poikilothermic fish, and is an occa-
60 sional human pathogen.

61 *M. marinum* serves as a model for several aspects of *M. tuberculosis* pathogenesis (14,
62 15). We previously reported the N-terminal acetylomes of *M. tuberculosis* and *M. marinum (16)* .
63 We found that 15% of *M. tuberculosis* proteins and 11% of *M. marinum* proteins are acetylated
64 at their N-termini (16). Approximately 16% of the mycobacterial proteome is acetylated at lysine
65 residues (17)**.** Acetylation mapped to metabolism, antibiotic resistance, iron uptake, and lipid syn-
66 thesis pathways critical to pathogenesis (17-20)**.**

67 The majority of predicted and established NATs belong to the GCN-5 Related N-acetyl-
68 transferase (GNAT) superfamily of enzymes (1). *M. tuberculosis* and *M. marinum* share 23 highly
69 conserved acetyltransferases (16), several of which are capable of acetylating more than one
70 type of target (21-31). Substrates of NATs cannot be predicted by protein sequence alone, requir-
71 ing that the substrates be determined empirically (*3*).

72 Early following infection, mycobacterial pathogens are taken up by professional phago-
73 cytes (32, 33). Following adaptation to the phagosome (34, 35), pathogenic mycobacteria use the
74 ESAT-6-system-1 (ESX-1) Type VII secretion system to lyse the phagosomal membrane and
75 promote interaction with the macrophage cytoplasm (36-40). Several mycobacterial lipids, includ-
76 ing phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL), promote optimal phagoso-
77 mal lysis (41-43), likely because these lipids are required for the wild-type levels of ESX-1 effector
78 proteins (44). Several ESX-1 proteins (EsxA, EspA, EspB, EspC, EspL, $EccA_1$, $EccB_1$, $EccCb_1$,

79 $EccE_1$, EsxH, and EspB), and PDIM/PGL biosynthesis proteins (Mas, PpsA, PpsE, Pks15/1,
80 PapA5 and DrrA) are N-terminally or lysine acetylated (16, 17, 45). We recently identified the
81 Emp1 (MMAR_1839) NAT as the sole enzyme that promotes N-terminal acetylation of EsxA in
82 *M. marinum in vivo (46)*. Here, we sought to identify highly conserved GNAT proteins between *M.*
83 *marinum* and *M. tuberculosis* that promote virulence through the ESX-1 and PDIM pathways,
84 leveraging genetics, biochemistry, proteomics, functional assays and models of infection.
85
86 **Results:**
87 **Several GNAT proteins contribute to *M. marinum* hemolytic activity***.* We generated *M. mari-*
88 *num* strains lacking individual genes with predicted or established NAT activity [Table 1, (46)],
89 and the corresponding complementation strains. We confirmed each strain using PCR (Figure
90 S1) followed by targeted DNA sequencing. ESX-1 and PDIM/PGL are required for optimal phag-
91 olytic activity of *Mycobacterium* (42, 47, 48). We tested the requirement of 19 conserved NATs
92 for *M. marinum* hemolytic activity, which can serve as a proxy for phagolytic activity. Six NATs
93 significantly impacted *M. marinum* hemolytic activity (Fig. 1A), while the remaining 13 NATs did
94 not significantly impact hemolysis (Fig. S2A). WT *M. marinum* lysed red blood cells (RBCs), while
95 the Δ$eccCb_1$ strain, which lacks a functional ESX-1 secretion system (37, 49), failed to lyse RBCs
96 (P<0.0001 relative to the WT strain) similar to the cell-free control (PBS). None of the *M. marinum*
97 strains lacking individual NATs phenocopied the Δ$eccCb_1$ strain (Figs. 1A and S2A). However,
98 the deletion of several individual NAT genes significantly reduced hemolysis compared to the WT
99 strain, including *argA, rimI*, *pat, MMAR_3205, MMAR_0332* and *mbtK* (all P<0.0001 vs the WT
100 strain*,* Fig. 1A). The *Δeis* strain was significantly more hemolytic than the WT strain (P=0.0032).
101 For all strains, except Δ*pat,* expression of the NAT gene of interest from the mycobacterial optimal
102 promoter significantly increased hemolysis compared to the deletion strain. From these data, we
103 conclude that several putative and established NATs are required for wild-type hemolytic activity
104 in *M. marinum.*

105 Several of the NATs were either established or predicted lysine acetyl transferases [KATs
106 in Fig 1A indicated by open bars, (21, 24, 26, 50-56)], and are the focus of this study. To test if
107 the predicted NAT impacted ESX-1 activity, we added an additional copy of the ESX-1 system to
108 the NAT deletion strains and tested for restoration of hemolysis. We introduced the pRD1-2F9
109 cosmid containing the extended RD1 locus from *M. tuberculosis*, which includes the majority of
110 the ESX-1 component and substrate genes (57), into the *M. marinum* strains lacking individual
111 KAT genes, as well as the WT and Δ*eccCb*$_1$ strains. The addition of a second copy of the ESX-1
112 locus to the WT strain did not significantly impact hemolysis (Fig. S2B). Because the *eccCb*$_{1MT}$
113 (MT = *M. tuberculosis*) gene is included on the pRD1-2F9 cosmid, the pRD1-2R9 plasmid com-
114 plemented the hemolytic activity of the Δ*eccCb*$_1$ strain. Interestingly, the pRD1-2F9 cosmid sig-
115 nificantly increased the hemolytic activity of the Δ*pat* and Δ*3205* strains (P<0.0001*,* relative to the
116 parent strains), supporting that Pat and MMAR_3205 impact hemolytic activity through the ESX-
117 1 system. The pRD1-2F9 cosmid did not significantly impact the hemolytic activity of the Δ*rimI,*
118 Δ*eis* or Δ*mbtK* strains, suggesting a specific connection between Pat and MMAR_3205*.*
119 We next tested if the deletion strains had altered production of PDIM, phenolic glycolipids
120 (PGLs) and tri-acyl glycerides (TAG). We extracted total lipids from the *M. marinum* strains lacking
121 individual KAT genes and their respective complementation strains and measured PGLs, PDIM
122 and TAG using TLC. The WT strain, but not the Δ*mas* strain produced PDIM, TAG (Fig. 1B, top
123 TLC) and PGL [bottom TLC, lane 1 and 2, (44, 46, 58-63)]. Both PDIM and PGL were reduced
124 and TAG levels were elevated in total lipids extracted from the Δ*mbtK* strain (lane 3). Expression
125 of the *mbtK* gene in the Δ*mbtK* strain increased PDIM and PGL, but did not reduce TAG levels
126 compared to the Δ*mbtK* strain (lane 4). Deletion or complementation of the other potential KAT
127 genes did not noticeably impact the lipids tested under these conditions (lanes 5-14). From these
128 data, we conclude that MbtK is required for the production of WT levels of PDIM and PGLs.
129

130 **Mycobactin deficiency is linked to reduced growth and PDIM and PGL production in *M.***
131 ***marinum.*** MbtK in is required for synthesizing mycobactin, a secreted siderophore required for
132 iron scavenging (55). In the absence of MbtK, *M. tuberculosis* growth *in vitro* was severely im-
133 pacted due to the loss of mycobactin production (55). Likewise, we found that the *ΔmbtK* strain
134 exhibited significantly reduced growth compared to the WT and complemented strains (Fig. S3A).
135 We next tested if the reduced growth and PDIM production in the Δ*mbtK M. marinum* strain could
136 be rescued by supplementing the Δ*mbtK* strain with ferric Mycobactin J. As shown in Figure S3C,
137 addition of mycobactin to the growth media restored growth of the Δ*mbtK* strain, similar to the
138 complemented and WT strains. We grew the *M. marinum* strains in the presence of a range of
139 mycobactin concentrations (from 2 µg/ml to 0.0002 µg/ml or in the absence of mycobactin) and
140 extracted total lipids. Using TLC, we found that PDIM and PGL production was restored to WT
141 levels in the presence of 2 µg/ml or 0.02 µg/ml mycobactin (Fig. S3C). 0.0002 µg/ml did not restore
142 PDIM or PGL production to the Δ*mbtK* strain From these data we conclude that the addition of
143 mycobactin J to the grown media trans-complemented the growth defect and PDIM/PGL produc-
144 tion of the Δ*mbtK* strain. These data support the idea that PDIM and PGL production was reduced
145 in the Δ*mbtK* strain due to a deficiency in iron scavenging.
146 MbtK, and its role in mycobactin synthesis, was previously linked to phospholipid homeo-
147 stasis (55). However, MbtK has not previously been linked to PDIM and PGL production. To gain
148 insight into the mechanisms underlying the phenotypes associated with the loss of MbtK we used
149 proteomics to measure the changes in protein levels. We generated cell-associated and secreted
150 protein fractions from the WT, Δ*mbtK,* and the Δ*mbtK*/p*mbtK* strains (Dataset S1) and measured
151 protein levels in each fraction using nUHPLC MS-MS/MS quantitative bottom-up proteomics (44,
152 64). The deletion of *mbtK* resulted in a significant increase in 15 proteins (Figure S4A), the ma-
153 jority of which are associated with three chromosomal regions associated with iron uptake, includ-
154 ing ESX-3 (EccB3 and MycP3), MMAR_2874, MMAR_2875, cyp278A1 and MMAR_2878, which
155 are colocalized on the chromosome, and MbtF, MbtE and MbtD, which are colocalized with MbtK

156 on the chromosome. The Cyp278A1 protein is annotated as being involved in heme uptake (65).
157 The additional genes are conserved hypothetical proteins. Expression of *mbtK* in the Δ*mbtK* strain
158 restored protein levels to those in the WT strain. We also measured the protein levels in the
159 secreted fractions. The levels of the Mbt proteins were increased in the secreted fraction from the
160 Δ*mbtK* strain as compared to the strains expressing *mbtK*. There was no significant change in
161 the secretion of any ESX proteins to explain the reduced hemolytic activity in Fig. 1A. These data
162 did not allow us to connect the loss of MbtK to the production of PDIM and PGL.
163
164 **Overexpression of *eis* restores virulence lipids to the Δ*mbtK* strain.** To understand the re-
165 quirement for MbtK in the lipid biosynthesis, we asked if constitutive expression of the other pu-
166 tative KAT genes could restore wild-type PDIM, PGL and TAG levels to the Δ*mbtK* strain. We
167 introduced each KAT gene into the Δ*mbtK* strain on an integrating plasmid, expressed from the
168 MOP promoter, and confirmed the presence of the plasmid in each strain by PCR (Fig. S5). The
169 expression of the *rimI, pat* or *MMAR_3205* genes did not detectably impact the levels of PDIM,
170 TAG or PGLs in the Δ*mbtK* strain (Fig. 2A, lanes 5-7). However, constitutive expression of the
171 *eis* gene specifically restored PDIM, TAG and PGL levels in the Δ*mbtK* strain (lane 8) to those in
172 the WT and complemented strains.
173 Eis is a N-acetyltransferase that is clinically linked to aminoglycoside resistance in *M. tu-*
174 *berculosis* (28, 66-71). To our knowledge, there are no known connections between the Eis and
175 MbtK acetyltransferases. We first considered if Eis restored lipid production simply because it was
176 the most similar NAT to MbtK. Based on structural predictions, MbtK and Eis proteins do not share
177 domains beyond the conserved GNAT domains (AlphaFold, Fig 2B). The RimI, Pat or
178 MMAR_3205 proteins that did not functionally complement the Δ*mbtK* also share the GNAT do-
179 main. At the amino acid level, Eis, RimI and MMAR_3205 are all ~20% identical to MbtK (Fig.
180 2C), and both MbtK1 and MMAR_3205 are more phylogenetically related KAT to MbtK the Eis
181 (Fig. 2D). Therefore, we hypothesized that Eis specifically restored PDIM/PGL production to the

182 Δ*mbtK* strain. Our proteomics analysis shows that expression of *eis* in the Δ*mbtK* strain restored
183 the levels of 12 of the 15 previously discussed proteins, with the exception of MMAR_2874,
184 MMAR_2875 and Cyp278A1, which are at the same genetic locus (Fig. S4). These data suggest
185 that *eis* partially complements the Δ*mbtK* strain.

186 *M. tuberculosis* required supplementation with iron during laboratory culture when the
187 $mbtK_{MT}$ gene was deleted (55). The Δ*mbtK* (*MMAR_3692*) *M. marinum* strain exhibited reduced
188 growth without the addition of supplemental iron compared to the WT strain (Fig. S3A). An orthol-
189 ogous *mbtK* gene, *MMAR_1587*, in *M. marinum* genome which may allow growth of the Δ*mbtK*
190 strain without additional iron. MMAR_1587 is 28.5% identical at the amino acid level, and is pre-
191 dicted to be structurally similar to MbtK (Fig. S6A) MMAR_1587 is 54.73% identical to the $MbtK_{MT}$
192 protein at the amino acid level, and is annotated as the MbtK ortholog on Mycobrowser (15).
193 Although the MMAR_3692 protein is 26% identical to $MbtK_{MT}$, the *MMAR_3692* gene is syntenic
194 with the $mbtK_{MT}$. As such, we refer to *MMAR_3692* as *mbtK,* and *MMAR_1587* as *mbtK1.* (Fig.
195 2D). *M. marinum* has an orthologous *eis* gene, *MMAR_0151,* which is annotated as *eis1* (65).
196 The Eis and Eis1 proteins are 31.44% identical, and are predicted to be structurally similar (Fig.
197 S6A). To further probe the interaction between MbtK and Eis, we constitutively expressed *mbtK1*
198 and *eis1* in the Δ*mbtK* strain. Constitutive expression of the *mbtK1* or *eis1* genes did not com-
199 pletely rescue PDIM or PGL levels in the Δ*mbtK* strain as visualized by TLC (Fig. S6B). These
200 data suggest that additional copies of MbtK or Eis are functionally redundant, and that Eis likely
201 specifically rescues PDIM, PGL and TAG production in the Δ*mbtK* strain.

202

203 **Eis N-terminally acetylates PapA5 *in vitro*.** The proteomics data did not provide an obvious
204 mechanism for how Eis restored PDIM and PGL production to the Δ*mbtK* strain. We demonstrated
205 that several proteins required for PDIM and/ or PGL production (PpsA, PapA5, Mas and PpsE,
206 Pks15/1) were N-terminally acetylated [Fig S7, Pink, (*1*)]. Although neither MbtK nor Eis have

207 reported to N-terminally acetylate proteins, we tested if MbtK and/or Eis could N-terminally acet-
208 ylate one or more biosynthetic proteins required for PDIM and PGL production.

209 It was previously established that the first six amino acids of a substrate protein were
210 sufficient for N-terminal acetylation *in vitro* (30, 72). We had N-terminal six AA peptides synthe-
211 sized based on the N-terminal peptides we previously measured using proteomics [Fig. 3A, (16)].
212 Only the Mas peptide contained a "K" residue, suggesting that we could measure N-terminal acet-
213 ylation of these peptides. The N-terminal peptides were used as substrates for *in vitro* acetylation
214 assays with the WT or inactive versions of MbtK (MbtKE161A, active site mutation as predicted
215 by UniProt) and Eis [EisY128A, proton donor active site mutant based on (29)] that we expressed
216 and purified from *E. coli* (Fig. S8). Transferring the acetyl group from Acetyl-CoA to a peptide
217 substrate results in a free thiol group on the resulting CoA molecule. The reaction of this free thiol
218 with 5, 5'-dithiobis-(2-nitrobenzoic acid) DTNB results in a colorimetric change that can be meas-
219 ured at 412nm. The resulting product has 1:1 stoichiometry with the acetylated peptide and can
220 be used to quantify protein acetylation (29, 73). The RimI NAT from *Salmonella* with the S18
221 substrate [(72, 74), Fig. S8] was a positive control for the reaction. Incubation of the PpsE
222 (P=0.0147) or PapA5 (P<0.0001) peptides with the WT MbtK resulted in significantly higher DTNB
223 production than incubation with the MbtKE161A protein (Fig. 3B). Incubation of the PpsA, Mas or
224 Pks15/1 peptides resulted in product formation that was not significantly different between the
225 active and inactive enzymes. These data support that MbtK may acetylate the N-terminal amino
226 acid of PpsE or PapA5.

227 We conducted the same experiment using Eis or EisY128A, a proton donor active site
228 mutant (29). Incubation of the PapA5 peptide with the WT Eis protein resulted in significantly
229 higher DTNB production than incubation with the Eis Y128A protein (P=0.0096, Fig. 3C). None of
230 the other peptides tested exhibited significantly reduced product formation between the WT and
231 dead enzymes. These data are consistent with the possibility that Eis may N-terminally acetylate
232 PapA5.

233 To directly test the acetylation of PpsE and PapA5 by MbtK and Eis, we used FIA mass
234 spectrometry. As shown in Figure 3D, MbtK did not N-terminally acetylate the PpsE peptide. Alt-
235 hough we detected the unacetylated peptide (left), we did not detect the acetylated peptide. We
236 likewise tested if Eis or MbtK could N-terminally acetylate the PapA5 peptide. The unacetylated
237 PapA5 peptide was detected when mixed with either Eis (Fig. 3E, top left) or MbtK (bottom left).
238 The acetylated PapA5 peptide was detected in the presence of Eis, but not MbtK (Fig. 3E, right).
239 We conclude that Eis, but not MbtK, N-terminally acetylates PapA5 *in vitro*. These data suggest
240 that *eis* expression in the Δ*mbtK* strain restores PDIM production by N-terminal acetylation of the
241 PapA5 protein (Fig. 3F). We attempted to measure N-terminal acetylation in the Δ*mbtK* strain,
242 and in the corresponding complementation and *eis* expression strain (Dataset S1). We were un-
243 able to measure the N-terminal peptide of PapA5 in these strains. We did not identify N-terminal
244 peptides whose acetylation in the Δ*mbtK* strain was significantly decreased and was restored by
245 both the *mbtK* and *eis* expression, or by expression of the *eis* gene. Potential candidates for
246 targets that did not reach significance are in (Fig. S4B). These data did not allow us to determine
247 if the N-terminal acetylation of PapA5 was promoted by *eis* expression in *M. marinum*.

248 We tested if *papA5* could restore PDIM/PGL production or growth to the *ΔmbtK* strain by
249 collecting total lipids and visualizing PDIM and PGL on TLCs. Expression of *papA5* did not in-
250 crease PDIM or PGL Levels (Fig. 3F, lanes 2 and 3). Expression of the *papA5* gene in the Δ*mbtK*
251 strain increased the levels of PDIM and PGLs in this strain, but not to WT levels (lanes 4 and 5,
252 and additional replicates in Fig S9). Together these data support the conclusion that although
253 PapA5 partially rescued the reduced PDIM/PGL levels in the Δ*mbtK* strain, it did not rescue
254 growth under the conditions tested.

255 **The requirement of MbtK for mycobacterial pathogenesis can be suppressed by Eis**
256 **or PapA5.** Because the Δ$mbtK_{MT}$ was attenuated for virulence in a mouse model of infection (55),
257 we tested if the Δ*mbtK* strain was attenuated in two models of mycobacterial infection. First, we
258 tested if *mbtK* was required for macrophage cytolysis using Ethidium homodimer (Eth-D1), which

259 is a membrane impermeable nucleic acid stain (*16*). If *M. marinum* infection perforates the mac-
260 rophage membrane, the cells stain red. We infected RAW 264.7 cells with *M. marinum* at an M
261 OI of 5, and counted the presence of red cells (Fig. 4A). Infection of RAW 264.7 cells with wild-
262 type *M. marinum* (P<0.0001), but not the Δ*eccCb*$_1$ strain, resulted in significant levels of cytolysis
263 compared to the uninfected cells. The Δ*mas* strain and the Δ*mbtK* strains resulted in significantly
264 less cytolysis than the WT strain (P<0.0001). The complementation strains significantly increased
265 cytolysis compared to the deletion strains (P<0.0001) but were still significantly lower than the
266 WT strain (P<0.0001and P=0.0090, respectively). The *ΔmbtK* strain expressing the *mbtKE161A*
267 gene did not significantly affect cytolysis compared to the Δ*mbtK* strain. From these data we con-
268 clude that the NAT activity of MbtK is required for macrophage cytolysis.

269 We tested macrophage cytolysis following infection with the Δ*mbtK* strain constitutively
270 expressing the *eis* or the *eisY128A* gene (Fig. 4B). Expression of the *eis* or *eisY128A* gene in the
271 Δ*mbtK* strain significantly increased cytolysis compared to the Δ*mbtK* strain (P<0.0001), similar
272 to the WT strain. Infection with the Δ*mbtK* strain expressing the *eisY128A* gene resulted in signif-
273 icantly reduced cytolysis compared to the *eis* gene (P=0.0001). We conclude that constitutive
274 expression of *eis* suppresses the loss of MbtK during macrophage infection, and that this sup-
275 pression requires the NAT activity of Eis.

276 We tested if constitutive expression of *papA5* would likewise restore cytolysis to the Δ*mbtK*
277 strain. Infection of RAW 264.7 cells with the Δ*mbtK* strain constitutively expressing *papA5* resulted
278 in significantly more cytolysis than the WT (P=0.0218) or Δ*mbtK* strains (P<0.0001, Fig. 4A). To
279 test if suppression of cytolysis by *papA5* expression was specific we added two controls. First,
280 constitutive expression of *papA5* in the Δ*mas* strain failed to restore cytolysis to the Δ*mas* strain.
281 Second, constitutive expression of the *rimI* NAT in the Δ*mbtK* strain failed to restore cytolysis to
282 the Δ*mbtK* strain. Collectively, our data support that acetylation of PapA5 by Eis bypasses the
283 loss of MbtK.
284

285 We tested if the orthologous *mbtK1* and *eis1* genes were essential for macrophage cytol-
286 ysis. Expression of the *mbtK1* gene partially restored cytolysis to the Δ*mbtK* strain (Fig. S7B)
287 Expression of the *eis1* gene in the Δ*mbtK* strain resulted in cytolysis levels significantly higher
288 than the WT strain (P<0.0001). Deletion of the *mbtK1* or *eis* genes significantly reduced macro-
289 phage cytolysis compared to the WT strain (P<0.0001), but cytolysis was not restored upon ex-
290 pression of *mbtK1* or *eis* in these strains. Deletion of the *eis1* gene resulted in a significant reduc-
291 tion of macrophage cytolysis compared to the WT strain, and this was significantly increased upon
292 expression of the *eis1* gene, though not to WT levels.

293 To complement the cellular model of infection, we tested the ability of *M. marinum* to kill
294 the waxworm, *Galleria mellonella* larvae*. G. mellonella* was previously established as a model for
295 mycobacterial infection (75-79). For each experiment, we infected 30 worms, each injected with
296 $1x10^7$ bacteria in a 5 µL volume. Experiments were performed in biological triplicate. As shown in
297 Figure 4B, WT *M. marinum* killed all of the infected *G. mellonella* by 7 days post-infection. In
298 contrast, infection with either the Δ*eccCb*$_1$ or Δ*mbtK* strains resulted in less killing, similar to the
299 uninfected worms. Deletion of *eis* did not significantly impact the killing of *G. mellonella.* Expres-
300 sion of *mbtK* restored the ability of the Δ*mbtK* strain to kill *G. mellonella,* similar to the WT strain.
301 Expression of either *eis* or *papA5* in the Δ*mbtK* strain partially restored the ability of *M. marinum*
302 to kill *G. mellonella.* Introduction of the pRD1-2F9 plasmid to the Δ*mbtK* strain or growing the
303 Δ*mbtK* strain in ferric mycobactin did not restore *G. mellonella* killing to the ΔmbtK strain (Fig.
304 S3B). Together, these data support that the expression of Eis or PapA5 suppresses the loss of
305 *mbtK* in *M. marinum.*

306

307 **Discussion:**

308 We sought to identify conserved GNAT containing proteins important for the ESX-1 and PDIM
309 virulence pathways. We found that the majority of GNAT proteins previously annotated as lysine
310 acetyltransferases were required for wild-type levels of hemolysis by *M. marinum.* We identified

311 several potential NATs required for hemolysis that could be rescued by the addition of a second
312 ESX-1 locus. These GNAT proteins will be followed up in future studies. Our data support a model
313 where mycobactin-mediated iron uptake promotes the PapA5-mediated biosynthesis of PDIM and
314 PGLs (80). In the absence of MbtK, the production of PDIM and PGLs is reduced compared to
315 the WT *M. marinum* strain. PDIM and PGL production was complemented by expression of either
316 *mbtK* or *in trans* by the addition of Mycobactin J to the growth media. Complementation was
317 dependent upon the acetyltransferase activity of MbtK, as expression of the *mbtKE161A* allele,
318 which does not have NAT activity, did not restore PDIM and PGL production. We showed that the
319 expression of *papA5* or the *eis* NAT in the Δ*mbtK* strain specifically suppressed the requirement
320 of MbtK in PDIM and PGL production. The simplest mechanism, which is supported by our *in vitro*
321 assays, is that Eis N-terminally acetylates PapA5, promoting the production of PDIM and PGL in
322 the absence of MbtK. Overall, our studies support a previously undiscovered link between MbtK,
323 iron uptake and the final step of PDIM and PGL biosynthesis (Fig. 5).

324 Our model is supported by several lines of evidence previously published in the literature.
325 It was previously established by Madigan et al that MbtK is required broadly for lipid and myco-
326 bactin production (55). MbtK is required for the synthesis of the lipid tail of the mycobactin sider-
327 ophore, but more broadly, the loss of MbtK and iron uptake in *M. tuberculosis* resulted in a deple-
328 tion of phospholipids during iron starvation (55). Although Madigan et al connected lipid produc-
329 tion to MbtK mediated siderophore iron uptake in *M. tuberculosis,* the PDIM and PGL lipids were
330 not readily measured using lipidomics approaches in this study. As such, the connection between
331 mycobactin dependent iron uptake and PDIM/PGL biosynthesis has not been previously investi-
332 gated. The connection between iron and PDIM/PGL biosynthesis could be direct. For example,
333 the PapA5 mediated synthesis step in PDIM biosynthesis could require iron. In support of this,
334 *papA5* overexpression or the addition of mycobactin restored PDIM/PGL biosynthesis in the
335 Δ*mbtK* strain. Alternatively, the connection between iron and PDIM/PGL biosynthesis could be
336 indirect. For example, it is possible that iron levels are a signal that regulate the production of

337 PDIM and PGL. Further experimentation is required to differentiate between these two possibili-
338 ties.

339 The mechanisms connecting MbtK to mycobacterial pathogenesis are unclear. Yet, it was
340 surprising that constitutive expression of the *papA5* or *eis* genes fully restored macrophage cytol-
341 ysis to the Δ*mbtK* strain. Infection of *G. mellonella* revealed that increasing PapA5 or Eis restored
342 virulence to the Δ*mbtK* strain, but the kinetics of killing differed from that of the WT strain. These
343 data support that perhaps expression of these genes restored PDIM/PGL production, but did not
344 fully complement the Δ*mbtK* strain. Further work is required to determine if constitutive expression
345 of *eis* or *papA5* restore more that the PDIM/PGL deficiency to the Δ*mbtK* strain.

346 In this study, we uncovered a link between Eis and PapA5. Eis is a bifunctional N-acetyl-
347 transferase that acetylates aminoglycoside antibiotics to inactivate them. Mutations that lead to
348 *eis* overexpression naturally occur in clinical isolates of *M. tuberculosis,* leading to kanamycin
349 resistance (28, 66-71). Numerous studies have reported inhibitors of Eis as a potential path to
350 prevent aminoglycoside resistance during *M. tuberculosis* infection (81-90). Eis reversibly acety-
351 lates lysine residues in the HupB protein, affecting chromosomal condensation in *M. tuberculosis*
352 (26). We showed that Eis N-terminally acetylates PapA5 *in vitro*, adding another potential activity
353 to this enzyme. We were unable to detect the N-terminal peptide of PapA5 in our proteomics
354 experiment. However, expression of *papA5* suppressed several phenotypes of the Δ*mbtK* strain
355 supporting this connection (Fig. 5). If Eis acetylates the N-terminus of PapA5 *in vivo*, this modifi-
356 cation must promote PapA5 activity, allowing suppression of the PDIM/PGL defect in the Δ*mbtK*
357 strain. Our data further support an emerging literature that NATs, including Eis, are multifunctional
358 (91).

359 Knobloch et al previously suggested that the orphan *mbtK* gene (*MMAR_1587*) was the
360 *M. marinum* ortholog of *M. tuberculosis* MbtK (92). Despite being located at the *mbt* locus,
361 *MMAR_3692* was not mentioned in this publication. In our study, we found that deletion of the
362 *MMAR_3692* gene resulted in reduced growth during standard culturing conditions. The growth

363 defect was significant, but unlike in *M. tuberculosis,* the deletion of *mbtK* did not abrogate growth.
364 Similar to *M. tuberculosis,* supplementation with mycobactin restored growth of the Δ*mbtK* strain
365 (55). Deletion of the *mbtK* gene, but not the *MMAR_1587* gene resulted in a measurable
366 PDIM/PGL defect. Likewise, expression of *MMAR_1587* in the Δ*mbtK* strain did not restore PDIM
367 levels. From our studies, we propose that the *MMAR_3692* gene is the *M. marinum* ortholog of
368 the *M. tuberculosis mbtK*. Our data also suggests that the two genes in *M. marinum* are function-
369 ally distinct.

370 In non-tubercular mycobacterial species, there are two functionally distinct eis genes. In
371 *M. abscessus,* these are annotated as Eis1Mab and Eis2mab (93). Overexpression of *eis2mab,*
372 but not *eis1mab,* resulted in resistance to Kanamycin. We likewise found that there are two po-
373 tential *eis* genes in *M. marinum.* Deletion of either *eis* gene attenuated *M. marinum* in a macro-
374 phage model of infection (Fig. S6C). Overexpression of *eis,* but not *eis1* in *M. marinum* resulted
375 in low level Kan resistance (Fig S10), supporting that robust literature linking Eis levels to amino-
376 glycoside resistance, and that the two *eis* genes are functionally distinct. Our macrophage infec-
377 tion data supports that overexpression of *eis1* restored cytolysis of the Δ*mbtK* strain to greater
378 than WT levels (Fig. S6). Yet, expression of *eis1* did not restore PDIM/PGL production to the
379 Δ*mbtK* strain. The mechanisms connecting Eis1 to MbtK need further experimentation.

380 Overall, this study revealed a new connection between iron scavenging and PDIM and
381 PGL biosynthesis that requires further investigation. We uncovered that the Eis NAT, which has
382 been widely linked to drug resistance in *Mycobacterium,* also has the potential to N-terminally
383 acetylate proteins, including those required for the production of the PDIM and PGL lipids. Our
384 data support that NATs cannot be characterized by the targets they modify, and likely play unex-
385 pected, interconnected and as of yet undefined roles in mycobacterial pathogenesis and physiol-
386 ogy.
387

388 **Methods:** A summary of the methods are found here. Where necessary, detailed methods are
389 provided in the Supplementary Information. *Mycobacterium marinum* strains were derived from
390 the *Mycobacterium marinum* M strain (ATCC BAA-535), and maintained as described previously.
391 In frame deletion strains were generated using allelic exchange, and can be found in TableS1.
392 Complementation plasmids, found in Table S2, were constructed using the primers in Table S3
393 using FastCloning. Site directed mutagenesis was performed as described previously (94). He-
394 molysis assays were performed as described. RAW 264.7 cells were maintained as described
395 and infected with *M. marinum* at an MOI of 5. Macrophage cytotoxicity were measured by meas-
396 uring Ethidium homodimer uptake as described. *G. mellonella* larval infections were performed
397 by injecting *M. marinum* at a concentration$1x10^7$/5µL. Thin-layer chromatography experiments
398 was performed as described in (46). Expression and purification of 6xHis-tagged proteins were
399 expressed in *E. coli,* and purified using Ni-NTA resin, followed by removal of the epitope tag using
400 TEV. Proteins were used to test activity in vitro against N-terminal peptides (Genscript) using 5,5-
401 dithio-bis-(2-nitrobenzoic acid) (DTNB) assays as (73) with minor modifications. Peptide acetyla-
402 tion following the DTNB assay was confirmed using MALDI. Bottom-up proteomics was performed
403 after labelling samples with deuterated acetyl groups as (16) to quantify protein levels and N-
404 terminal acetylation. Label-free quantification (LFQ) was used for differential protein expression
405 analysis, and data were processed using R (v4.3.2). The code can be found on github
406 (https://github.com/Champion-Lab/3692). Rawdata is available through MassIVE and The Prote-
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Figure Legends

Figure 1. At least four Lysine acetyltransferases are required for WT levels of hemolytic activity in *M. marinum.* A. Hemolytic activity of *M. marinum* strains lacking genes encoding predicted NATs. The putative/ known KATs are not filled. -, + refers to absence or presence of complementation plasmid, which gene of interest expressed from the mycobacterial optimal promoter. Significance relative to the WT strain; Brackets report significance between the deletion and complemented strains. **** $P<0.0001$, *** $P=0.0004$, 0.0002, * $P=0.0250$, 0.0298, ** $P=0.0032$ **B.** TLC analysis of total lipids from the indicated *M. marinum* strains. 6 µl of total lipids were analyzed in each experiment. The solvents used to visualize the indicated lipid species are indicated. The results are representative of at least three biological replicates.

Figure 2. Overexpression of Eis functionally restores PDIM production in the Δ*mbtK* strain **A.** TLC analysis of total lipids from the indicated *M. marinum* strains. The NAT genes in lanes 4-8 were constitutively expressed in the ΔmbtK strain using the mycobacterial optimal strain. 6 µl of total lipids was separated for each strain. The indicated solvents and lipids visualized are indicated. The TLC is representative of at least three biological replicates. **B.** Overlay of the predicted structures for MbtK and Eis NATs. The PDB files used were: https://alphafold.ebi.ac.uk/files/AF-B2HMD6-F1-model_v3.pdb and https://alphafold.ebi.ac.uk/files/AF-B2HM88-F1-model_v3.pdb. The structures were overlaid using PyMOL v.2.4.0. **C**. % amino acid identify of the NATs from panel A. Protein sequences were obtained from Mycobrowser (65), and aligned using Clustal Omega (95). **D.** Clustering by phylogenetic neighbor joining tree without distance corrections, generated based on protein alignments generated by Clustal Omega.

439 **Figure 3. PapA5 is N-terminally acetylated by Eis *in vitro.* A.** Table of peptides from proteins
440 in the PDIM/PGL pathways. B. DTNB assay measuring the production of TNB (412nm) upon the
441 acetylation of the PpsA, PpsE, Mas, Pks15/1 and PapA5 peptides by **B.** WT or inactive MbtK and
442 **C.** the RimI protein from *Salmonella typhimurium* with the S18 N-terminal peptide (ARYFRR).
443 DTNB assays were performed in technical triplicate. The data represent the mean product for-
444 mation from all three experiments. Significance was determined using a Student's t-test between
445 the active and inactive forms of each NAT protein. B.* P=0.0417, **** P<0.0001 C. ** P=0.0096**.**
446 **D.** Flow injection analysis of the N-terminal peptide of PpsE with purified MbtK protein. Left panel:
447 peaks corresponding to the unacetylated N-terminal peptide of PpsE. Right panel: mass range
448 corresponding to the acetylated version. **E.** Flow injection of the N-terminal peptide of PapA5
449 incubated with either purified Eis protein or purified MbtK protein. Top left panel: peaks corre-
450 sponding to unacetylated PapA5. Top right: the acetylated version of PapA5 when incubated with
451 Eis. Bottom left panel: peaks corresponding to unacetylated PapA5, Bottom right panel: the same
452 mass range corresponding to the acetylated version of PapA5. **F.** TLC on total lipids from *M.*
453 *marinum.* 6µl total lipid loaded into each lane, PDIM run 3x consecutively in the solvent indicated,
454 PGLs run once in the solvent indicated. PDIM lipids visualized by phosphomolybdic acid and
455 subsequent charring, PGL lipids visualized by 1% a-naphthol and subsequent charring. The TLC
456 is representative of 3 biological replicates, the remaining 2 are shown in Fig. S9.
457
458 **Figure 4. The requirement of MbtK for mycobacterial pathogenesis can be suppressed by**
459 **constitutive expression of Eis or PapA5. A**. RAW 264.7 cells infected with *M. marinum* strains
460 at an MOI of 5. The data is the average of at least 3 independent infections, each performed in
461 biological triplicate. **** P<0.0001, *** P=0.0001, ** P=0.0218, #### P<0.0001. **B.** *Galleria*
462 *mellonella* infected with the *M. marinum* strains indicated. Larvae were injected with $1x10^7$ bacte-
463 ria into the right hindmost proleg. Larvae were infected in 3 groups of 10, for a total of 30 larvae
464 per strain per infection. Data is representative of at least 3 biological replicates, each performed

465 in technical triplicate. Statistical analysis was performed as in (79). Survival curves were com-
466 pared using a log-rank Mantel-Cox test, $P<0.0001$.
467
468 **Figure 5. Model of Interactions between MbtK, Eis and PDIM biosynthesis.** This study re-
469 vealed that MbtK, likely through siderophore production, is required for the production of PDIM
470 and PGL in *M. marinum.* This requirement was complemented in trans by the addition of Myco-
471 bactin J. Constitutive expression of either Eis, or PapA5 specifically suppressed the loss of
472 PDIM/PGL production in the absence of MbtK, suggesting that the N-terminal acetylation of
473 PapA5 could promote the restoration of PDIM/PGL synthesis in the Δ*mbtK* strain.
474
475 **Table Legend**
476 **Table 1. Table of the *M. marinum* genes with their orthologs from *M. tuberculosis* and their**
477 **commonly used names.**

M. marinum gene	*M. tuberculosis* gene	Name
MMAR_1067	*Rv0730*	
MMAR_1968	*Rv2747*	ArgA
MMAR_4519	*Rv0995*	RimJ
MMAR_1839	*Rv2867c*	Emp1
MMAR_1882	*Rv2851c*	ElaA
MMAR_2463	*Rv1653*	ArgJ
MMAR_0522	*Rv0262c*	Aac
MMAR_1123	*Rv3420c*	RimI
MMAR_4496	*Rv0998*	mPat
MMAR_4889	*Rv0802c*	
MMAR_3205	*Rv2170*	
MMAR_0332	*Rv0133*	
MMAR_1341	*Rv3216*	
MMAR_3740	*Rv2416c*	Eis
MMAR_2836	*Rv3225c*	
MMAR_2615		
MMAR_0151		Eis1
*MMAR_3692**	*Rv1347c*	MbtK
MMAR_2744		

478

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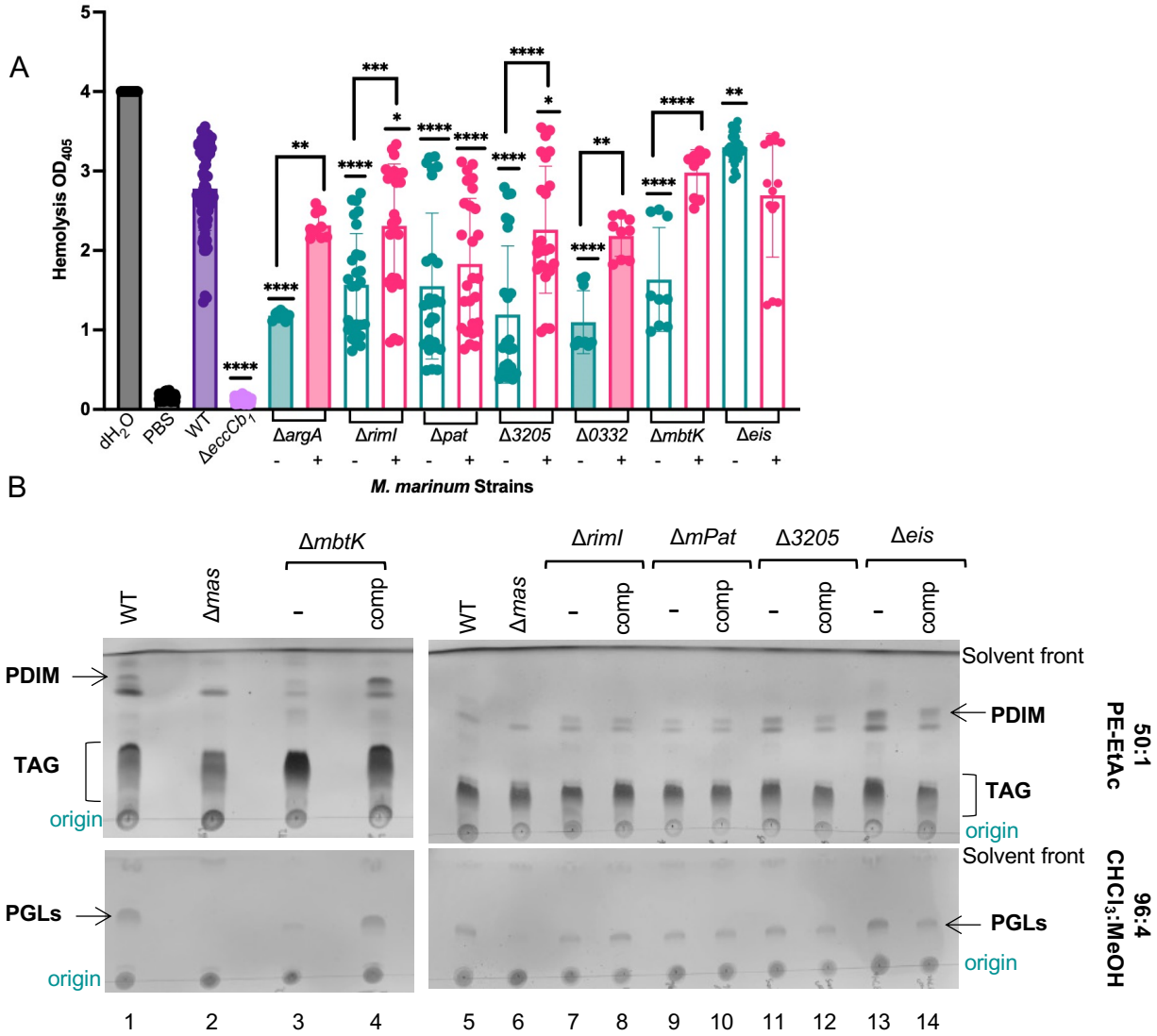
731 Figure 1.



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735 Figure 2.

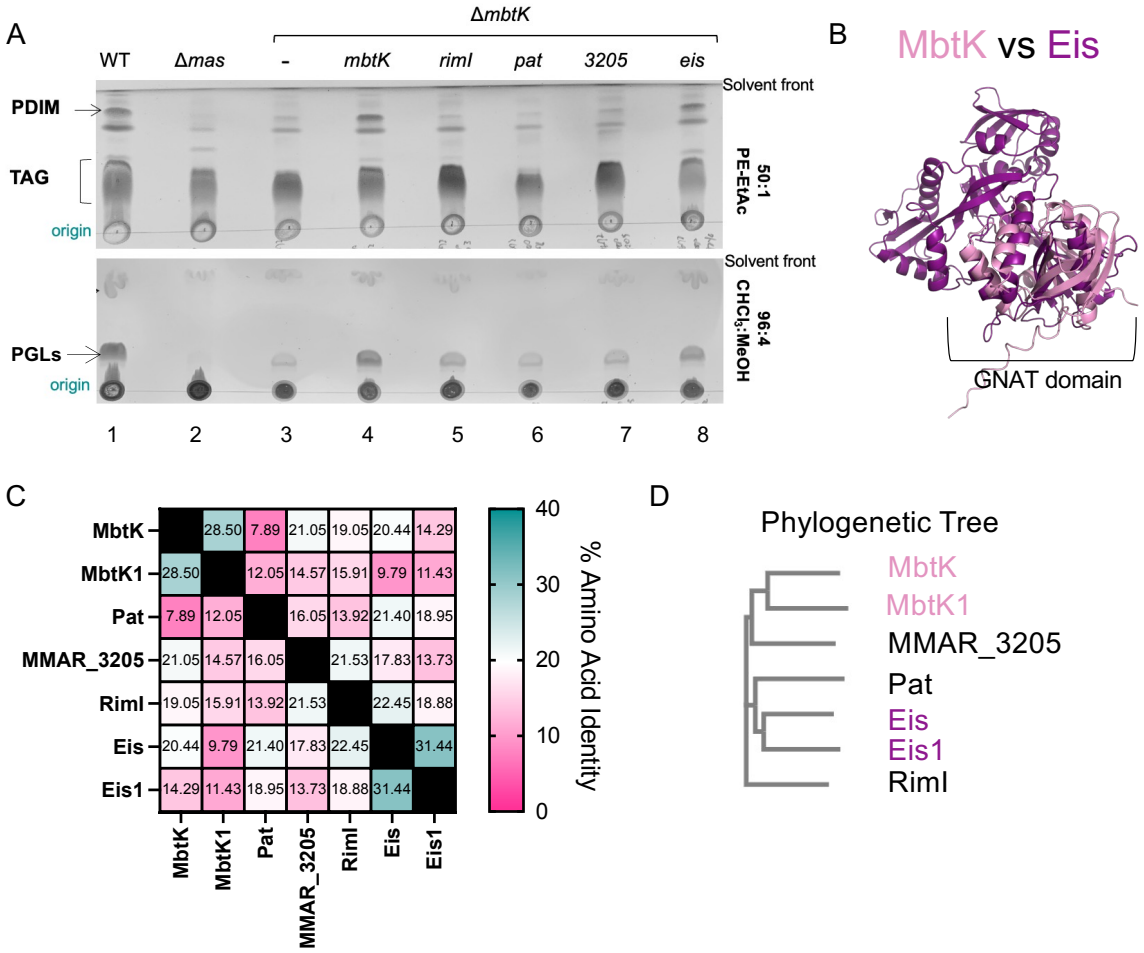

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Figure 3.

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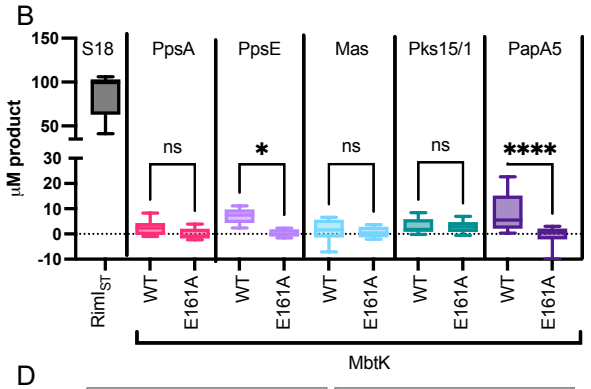
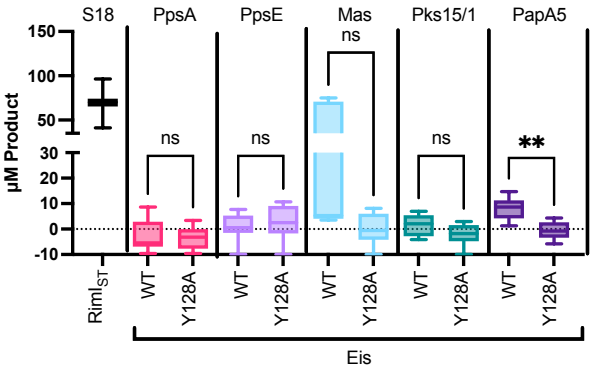
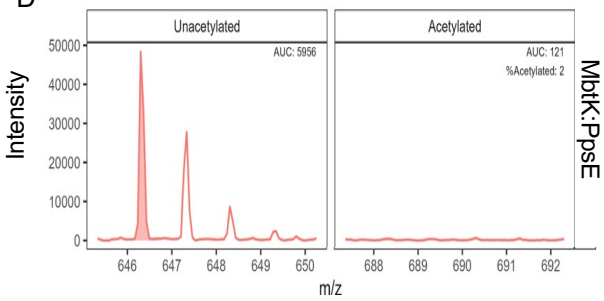
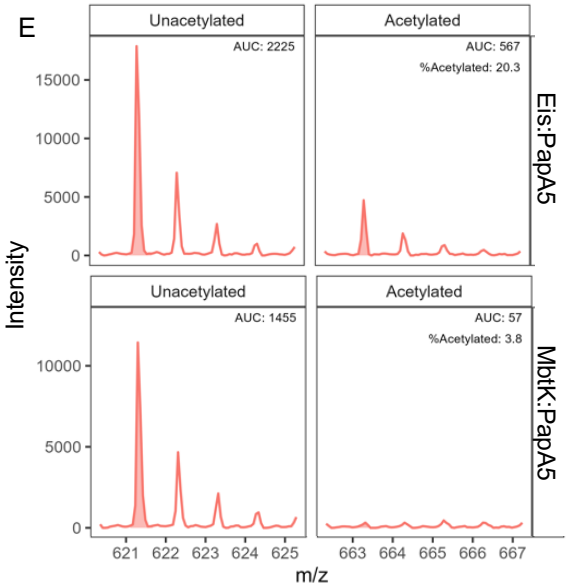
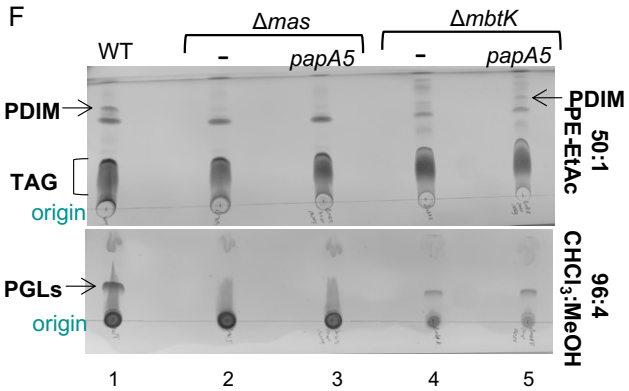
Protein	Nt-Peptide Sequence	Pathway
PpsA	TPSIGG	PGL/PDIM
PpsE	STIPDN	PGL/PDIM
Pks15/1	TTSGES	PGL
Mas	MQKPAT	PGL/PDIM
PapA5	MFPGAV	PGL/PDIM











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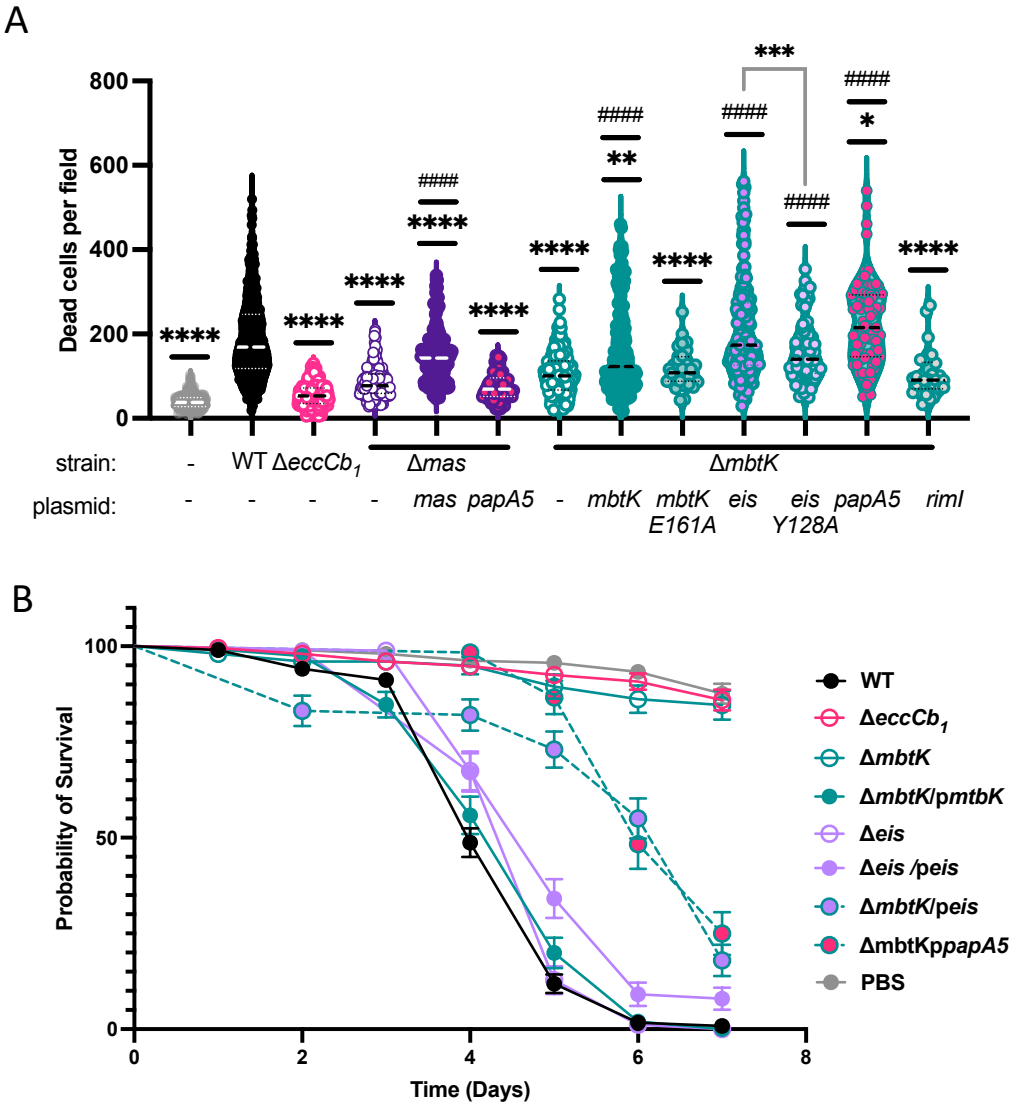

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745 Figure 5.
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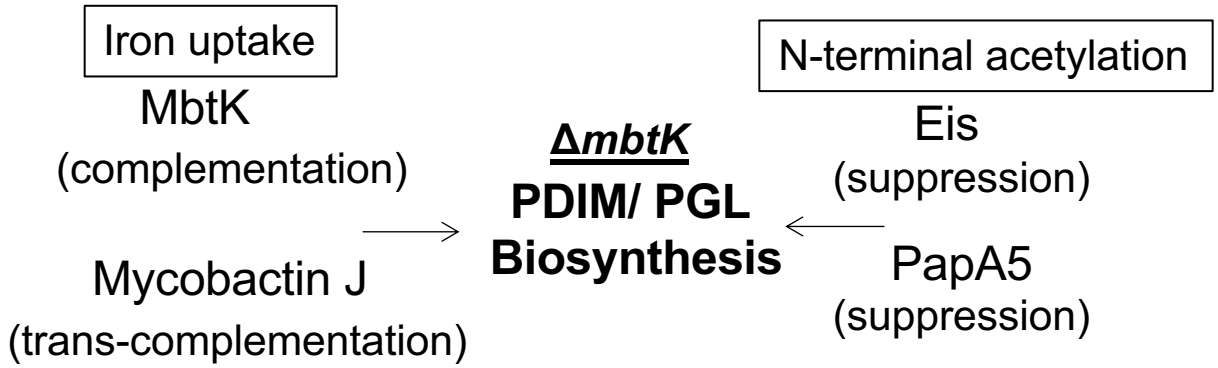

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