Cytokinin signaling in *Mycobacterium tuberculosis*

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Keywords: *Mycobacterium tuberculosis*, cytokinins, cell envelope, proteasome, Pup, antibiotic resistance

Summary

It was reported the human-exclusive pathogen *Mycobacterium (M.) tuberculosis* secretes cytokinins, which previously had only been known as plant hormones. Cytokinins are adenine-based signaling molecules in plants that have never been shown to participate in signal transduction in other kingdoms of life. Here, we show that cytokinins induce the strong expression of the *M. tuberculosis* gene, Rv0077c. We found that a TetR-like transcriptional regulator, Rv0078, directly repressed expression of the Rv0077c gene. Strikingly, cytokinin-induced expression of Rv0077c resulted in a loss of acid-fast staining of *M. tuberculosis*. Although acid-fast staining is thought to be associated with changes in the bacterial cell envelope and virulence, Rv0077c-induced loss of acid-fastness did not affect antibiotic susceptibility or attenuate bacterial growth in mice. Collectively, these findings show cytokinins signal transcriptional changes that affect the *M. tuberculosis* cell envelope, and that cytokinin signaling is no longer limited to the kingdom plantae.

47 **Introduction**

48 *M. tuberculosis* is the causative agent of tuberculosis, one of the world's leading causes
49 of mortality(WHO, 2017). For this reason, researchers are eager to identify pathways
50 that could be targeted for the development of new therapeutics to treat this devastating
51 disease. Among the current prioritized targets is the mycobacterial proteasome. *M.*
52 *tuberculosis* strains with defects in proteasome-dependent degradation are highly
53 attenuated in mice, partly because they are sensitive to nitric oxide (NO) (Cerda-Maira
54 *et al.*, 2010, Darwin *et al.*, 2003, Gandotra *et al.*, 2010, Gandotra *et al.*, 2007,
55 Lamichhane *et al.*, 2006, Lin *et al.*, 2009). The NO-sensitive phenotype of mutants
56 defective for proteasomal degradation has been attributed to a failure to degrade an
57 enzyme called Log (Lonely guy), a homologue of a plant enzyme involved in the
58 synthesis of a family of N^6-substituted adenine-based molecules called cytokinins
59 (Samanovic *et al.*, 2015). The accumulation of Log in *M. tuberculosis* results in a
60 buildup of cytokinins, a break down product of which includes aldehydes that effectively
61 sensitize mycobacteria to NO (Samanovic *et al.*, 2015).

62 While we determined that a lack of proteasome-dependent degradation results in
63 cytokinin accumulation, we were left with more questions, namely, what is the function
64 of cytokinin production by *M. tuberculosis*? In plants, cytokinins are hormones that
65 regulate growth and development (Sakakibara, 2006). In addition, bacterial plant
66 pathogens and symbionts use cytokinins to facilitate the parasitism of their plant hosts
67 (Frebort *et al.*, 2011). Outside of the laboratory, *M. tuberculosis* exclusively infects
68 humans and is not known to have an environmental reservoir; therefore, it is unlikely *M.*
69 *tuberculosis* secretes cytokinins to modulate plant development. Instead, we

70 hypothesized that *M. tuberculosis*, like plants, uses cytokinins to signal intra-species
71 transcriptional changes to its benefit. Here, we show that cytokinins induce the
72 transcription of a gene of unknown function. Moreover, we identified and characterized
73 a TetR-like regulator that represses the expression of this gene. While we have not yet
74 identified an *in vivo* phenotype associated with this cytokinin-inducible gene, we found
75 that its expression altered the cell envelope of *M. tuberculosis*, changing its staining
76 properties. Collectively, these studies provide a foundation to characterize cytokinin
77 signaling in *M. tuberculosis* and other cytokinin-producing bacterial species.
78
79 **Results**
80
81 **Cytokinins induce the specific and high expression of Rv0077c in *M. tuberculosis***
82 To test if a cytokinin (CK) could induce gene expression in *M. tuberculosis*, we grew
83 wild type (WT) *M. tuberculosis* H37Rv to mid-logarithmic phase and incubated the
84 bacteria for five hours with N^6-(Δ^2-isopentenyl)adenine (iP), one of the most abundantly
85 produced cytokinins in *M. tuberculosis* that is also commercially available(Samanovic *et*
86 *al.*, 2015) (**see Materials and Methods--table supplement 1**). Using RNA sequencing
87 (RNA-Seq), we discovered the expression of four genes, Rv0076c, Rv0077c, Rv0078,
88 and *mmpL6*, was significantly induced upon iP treatment compared to treatment with
89 the vehicle control (dimethylsulfoxide, DMSO) (**Figure 1A--table supplement 2**).
90 Rv0077c is conserved among many mycobacterial species, while Rv0076c and Rv0078
91 are present only in several mycobacterial genomes (**Figure 1B**) (Lechat *et al.*, 2008).
92 Notably, *M. smegmatis*, a distant, non-pathogenic relative of *M. tuberculosis*, has a

93 weak homologue of Rv0077c and no conspicuous Rv0078 homologue (**Figure 1B**).
94 *mmpL6* is one of 13 *mmpL* (mycobacterial membrane protein large) genes in *M.*
95 *tuberculosis*. In strain H37Rv, *mmpL6* is predicted to encode a 42 kD protein with five
96 trans-membrane-domains and is truncated compared to the same gene in ancestral
97 tuberculosis strains (Brosch *et al.*, 2002). Thus, it is unclear if *mmpL6* encodes a
98 functional protein in strain H37Rv.

99 Rv0077c was by far the most strongly induced gene in *M. tuberculosis* upon iP
100 treatment therefore we chose it for follow-up studies. We raised polyclonal antibodies to
101 recombinant Rv0077c protein and showed that protein levels were increased in *M.*
102 *tuberculosis* treated with iP for 24 hours (**Figure 1C,** first two lanes). Rv0077c was
103 barely detectable in cell lysates of bacteria that had not been incubated with iP and was
104 undetectable in a strain with a transposon insertion mutation in Rv0077c (**Figure 1C,**
105 center two lanes). Rv0077c protein was restored to WT levels in the mutant upon
106 complementation with an integrative plasmid encoding Rv0077c expressed from its
107 native promoter (**Figure 1C,** last two lanes). We also found a dose-dependent induction
108 of Rv0077c production using iP concentrations from 1 nM to 100 μM (**Figure 1D**).

109 We next synthesized and tested if the most abundantly produced cytokinin in *M.*
110 *tuberculosis*, 2-methyl-thio-iP (2MeSiP) (Samanovic *et al.*, 2015), could also induce
111 Rv0077c production. 2MeSiP strongly induced Rv0077c production (**Figure 1E,** lane 4).
112 Importantly, we did not observe induction of Rv0077c when we incubated the bacteria
113 with the appropriate cytokinin riboside (R) precursors iPR or 2MeSiPR (**Figure 1E,**
114 lanes 3 and 5). Similarly, adenosine monophosphate (AMP) or the closely related
115 molecule adenine could not induce Rv0077c expression (**Figure 1E,** lanes 6 and 7). We

116 hypothesized while adenine could not induce Rv0077c expression, at high enough
117 concentrations it could possibly inhibit Rv0077c induction by competing with cytokinin
118 for access to a transporter or receptor. Indeed, adenine reduced the induction of
119 Rv0077c by iP in a dose-dependent manner (**Figure 1F**).
120

121 **Identification of an operator for the TetR-like transcriptional repressor Rv0078**

122 Rv0077c is divergently expressed from Rv0078; the proposed translational start codons
123 for these genes are separated by 61 base pairs therefore we hypothesized that Rv0078
124 encodes a repressor of Rv0077c expression. We identified the promoter regions for
125 each gene by performing rapid amplification of 5' complementary DNA ends (5'RACE)
126 analysis for Rv0077c and Rv0078 and determined the likely start of transcription for one
127 gene was within the 5' untranslated region of the other gene (**Figure 2A**). We sought to
128 identify an operator sequence by using an electrophoretic mobility shift assay (EMSA,
129 **Figure 2B**). We narrowed down a putative Rv0078 binding site to a region overlapping
130 the proposed starts of transcription (+1) of both Rv0077c and Rv0078 (**Figure 2A**, red
131 box). TetR-like regulators generally bind to inverted repeat sequences; we identified a
132 21-base pair (bp) sequence within -14/+19 of Rv0077c containing two 10-bp inverted
133 repeat sequences. Mutagenesis of the probe in the repeat sequences disrupted Rv0078
134 binding to the DNA (**Figure 2B**). Importantly, the binding sequence overlaps the
135 putative transcriptional start sites of both genes, suggesting that expression of both
136 genes is repressed by Rv0078. Notably, the addition of iP did not result in the release of
137 Rv0078 from the DNA probe (**Figure 2B--figure supplement 1**). Therefore, cytokinins
138 do not appear to directly bind to Rv0078 to induce gene expression.

139 Based on the 5'RACE analysis we were able to delete and replace most of the
140 Rv0078 gene with the hygromycin-resistance gene (*hyg*) without disrupting the
141 promoter of Rv0077c in *M. tuberculosis* H37Rv. A ∆Rv0078::*hyg* strain displayed
142 constitutively high expression of Rv0077c irrespective of the presence of cytokinin,
143 supporting a model where Rv0078 directly represses Rv0077c expression (**Figure 2C**).
144 A single copy of Rv0078 expressed from its native promoter restored iP-regulated
145 control of Rv0077c in this strain (**Figure 2C,** lanes 5 and 6). Interestingly, in the process
146 of making the complementation plasmid, we acquired a random mutation (likely
147 generated during PCR) in Rv0078 that changed a tryptophan to arginine (W100R); this
148 allele was unable to complement the ∆Rv0078::*hyg* strain (**Figure 2C,** lanes 7 and 8).
149

150 **Two Rv0078 dimers bind to one operator**

151 To gain an understanding of how Rv0078 represses gene expression, we solved the
152 crystal structure of Rv0078 to 1.85 Å by single-wavelength anomalous dispersion
153 method. As previously reported, Rv0078 forms dimers resembling other TetR-like
154 proteins (Orth *et al.*, 2000, Wohlkonig *et al.*, 2017). Unlike the canonical TetR binding
155 site, *tetO*, which is 15 bp long, the Rv0078 binding site is 21 bp long suggesting Rv0078
156 binds to DNA differently than TetR. We performed an isothermal titration calorimetry
157 (ITC) experiment and determined that the apparent K_D of Rv0078 for operator DNA was
158 357 nM (**Figure 3A**). The estimated stoichiometry of DNA duplex to Rv0078 dimer was
159 0.66, suggesting that two Rv0078 dimers bind to one operator sequence.

160 We next co-crystallized Rv0078 with a 23-bp DNA fragment that was extended
161 one base pair at each end of the 21-bp operator sequence identified in Figure 2 (-8/+13

162 of Rv0077c) and solved the structure to 3.0 Å resolution (**Table supplement 3**). As
163 predicted by the ITC experiments, we observed two Rv0078 dimers bound to a DNA
164 duplex where each dimer bound on opposite sides of the DNA (**Figure 3B**). The top or
165 "central" dimer bound the DNA palindrome symmetrically while the bottom or
166 "peripheral" dimer bound to DNA off-center, staggered from the central dimer binding
167 site by seven base pairs. Thus, the longer-than-canonical (i.e., *tetO*) binding site is
168 required for accommodating two Rv0078 dimers, unlike TetR, which binds to *tetO* as a
169 single dimer.

170 Rv0078 has an N-terminal DNA-binding domain (DBD) and a C-terminal ligand-
171 binding domain (LBD) (**Figure 3B-D**). The DBD is comprised of three α-helices (α1 -
172 α3), and within this domain, α2 and α3 form a helix-turn-helix (HTH) motif that directly
173 contacts DNA. The LBD is formed by six α-helices (α4 - α9). The four Rv0078 protein
174 structures within a complex were nearly the same; the root mean square deviation
175 (rmsd) ranged from 0.07 Å to 0.17 Å in a pair-wise superimposition. The Rv0078 dimer
176 structure is held together by α8 and α9 of each monomer, forming a four-helix bundle.
177 The two dimer structures were highly similar with an rmsd of 0.14 Å. DNA binding
178 induced significant conformational changes across the Rv0078 dimer structure, with an
179 rmsd of 2.13 Å when compared to the DNA-free dimer. In particular, the two α3 helices
180 move towards each other by ~6 Å, in order to reduce their distance to 36.4 Å and fit in
181 the DNA major grooves (**Figure 3C**). In the LBD, the ligand entry between helices α4
182 and α5 is open and the ligand-binding pocket is empty. It appears that changes initiated
183 in the DBD regions upon binding to the DNA are transmitted to the LBD via the DBD-

184 LBD interface that involves extensive interactions, including two salt bridges and six H-
185 bonds (**Figure 3D**).

186 The ligand-binding pocket of Rv0078 is largely hydrophobic. Within this pocket,
187 we observed an elongated density resembling a long aliphatic chain of a fatty acid
188 (**Figure supplement 2**). Gas chromatograph-mass spectrometry of the compounds
189 extracted from Rv0078 purified from *E. coli* revealed fatty acids commonly found in this
190 organism, and a palmitate molecule fit well with the electron density (**Figure**
191 **supplement 2A-E**). A fatty acid carboxylate formed a hydrogen bond with Rv0078 Glu-
192 70 (**Figure 3E--figure supplement 2C**), and the long alkyl chain had numerous
193 hydrophobic interactions within the extended ligand pocket. When we purified Rv0078
194 under denaturing conditions and refolded the protein to remove the lipid, we observed
195 the same EMSA results as we observed with protein purified under native conditions
196 (data not shown), suggesting this fatty acid is unlikely to be the native Rv0078 ligand;
197 however, these data may suggest that the native ligand is fatty acid-like. Interestingly,
198 we found that Trp100 faces the ligand-binding pocket (**Figure 3E**), which suggests
199 Trp100 interacts with the natural ligand. This hypothesis is supported by our data
200 showing an $Rv0078_{W100R}$ mutant was unable to complement the Rv0078-deletion mutant
201 strain (**Figure 2C, lane 8**).

202 In addition to characterizing the LBD, we identified nine amino acids that were
203 important for interacting with DNA (**Fig. 4A-E**). Specifically, the hydroxyl groups of
204 Thr37, Thr47, and Tyr52 interacted strongly with DNA phosphates at a distance of 2.6
205 Å. Arg48 is the only residue that interacted with DNA by recognizing a guanine base at
206 a distance of 2.6 Å. To further examine the importance of these residues, we introduced

207 single amino acid substitutions (T37V, T47V, R48M, and Y52F) or double mutations
208 (T47V, R48M) into Rv0078 and performed EMSA assays. While all of the mutant
209 proteins were soluble and behaved like the WT protein in solution, $Rv0078_{R48M}$ did not
210 bind to DNA, and the other mutant proteins bound to DNA with reduced affinities
211 (**Figure 4F**).

212 To examine the DNA sequence specificity of Rv0078, we synthesized three
213 EMSA probes by changing the Rv0078 Arg48-interacting guanines to adenines (G-to-A)
214 in the central dimer binding region (probe S1), the peripheral dimer-binding region
215 (probe S2), or both dimer-binding regions (probe S3). These G-to-A substitutions either
216 abolished or compromised Rv0078 binding to DNA, affirming the critical role of
217 guanines in the binding site (**Figure 4G**). Notably, substitutions in probe S1 entirely
218 abolished Rv0078 binding, while substitutions in S2 retained partial gel shift at a high
219 concentration (white arrowhead in **Figure 4G**). This observation suggests that the
220 binding of the two Rv0078 dimers is cooperative, with the central dimer likely the first
221 one to bind to DNA.

222

223 **Constitutive expression of Rv0077c does not affect antibiotic susceptibility or**
224 **virulence in mice**

225 During our ongoing studies, a report was published on the identification of a small
226 molecule of the spiroisoxazoline family, SMARt-420, which strongly induces the
227 expression of the Rv0077c orthologue *bcg_0108c* in *M. bovis* Bacille Calmette-Guerin
228 (Blondiaux *et al.*, 2017). Using x-ray crystallography and surface plasmon resonance
229 techniques, the authors of this study found SMARt-420 binds to Rv0078 to derepress

230 binding from the Rv0077c promoter (Blondiaux *et al.*, 2017, Wohlkonig *et al.*, 2017).
231 SMARt-420 was identified in a search for compounds that boost the efficacy of the
232 second-line tuberculosis drug ethionamide (ETH). ETH is a pro-drug that is activated by
233 the mono-oxygenase EthA, which transforms ETH into highly reactive intermediates.
234 Activated ETH and nicotinamide adenine dinucleotide form a stable adduct, which binds
235 to and inhibits InhA, an essential enzyme needed for mycolic acid synthesis in
236 mycobacteria (DeBarber *et al.*, 2000, Vannelli *et al.*, 2002). While spontaneous
237 inactivating mutations in *ethA* result in resistance to ETH, it was proposed that the
238 induction of Rv0077c expression could bypass the need for EthA and transform ETH
239 into its toxic form (Blondiaux *et al.*, 2017). Based on this study, we predicted that an
240 Rv0078 mutant of *M. tuberculosis*, which expresses high levels of Rv0077c, should be
241 hypersensitive to ETH compared to the parental strain H37Rv. However, we observed
242 either little to no significant change in the 50% minimum inhibitory concentration (MIC_{50})
243 of ETH between the WT and ∆Rv0078::*hyg* strains (**Table supplement 4**). We also
244 tested if the constitutive expression of Rv0077c changed the susceptibility of *M.*
245 *tuberculosis* to other antibiotics, including two cell wall synthesis inhibitors. We
246 observed no differences in the MIC_{50} of these antibiotics between the WT and
247 ∆Rv0078::*hyg* strains (**Table supplement 4**).

248 We also tested if either the Rv0077c or Rv0078 mutant had growth defects *in*
249 *vivo* compared to the WT H37Rv strain. We infected C57BL/6J mice by a low-dose
250 aerosol route with the parental, mutant, and complemented mutant strains, as well as
251 with the Rv0078 mutant transformed with the $Rv0078_{W100R}$ allele. Interestingly, none of
252 the strains revealed a difference in growth or survival compared to WT *M. tuberculosis*

253 in mice as determined by the recovery of colony forming units (CFU) from the lungs and
254 spleens (**Figure 5--figure supplement 3**).
255
256 **Expression of Rv0077c alters acid-fast staining of *M. tuberculosis***
257 To gain insight into the function of Rv0077c, we performed metabolomics analysis of
258 strains expressing Rv0077c to potentially determine how its presence alters bacterial
259 physiology. We prepared total cell lysates of WT and Rv0077c mutant strains treated
260 with or without 100 μM iP for 24 hours (see Materials and Methods). From a total of 337
261 detectable metabolites, we observed a significant change in 24 molecules after the
262 addition of iP to WT *M. tuberculosis*. Seventeen metabolites showed a consistent
263 difference between samples in which iP was added and these changes disappeared in
264 an Rv0077c-disrupted strain, suggesting the changes were specifically due to the
265 presence of Rv0077c (**Table supplement 4**, highlighted in yellow). We observed an
266 increased abundance of several phospholipids and a decrease in a major precursor of
267 peptidoglycan, N-acetyl-glucosamine-1-phosphate (GlcNAc1P). We therefore
268 hypothesized that Rv0077c modified one or more components of the cell envelope.
269 Microscopic examination of Ziehl-Neelsen (ZN) stained WT *M. tuberculosis* treated with
270 iP showed a loss of acid-fast staining (**Figure 6**, panel a compared to b), and this
271 phenotype depended on the presence of Rv0077c (**Figure 6**, panel c compared to d).
272 Complementation of the mutation with Rv0077c alone restored the iP-induced loss of
273 acid-fast staining (**Figure 6**, panels e and f). Deletion of Rv0078 resulted in a
274 constitutive loss of staining, irrespective of the presence of iP (**Figure 6**, panels g and
275 h). Complementation of the Rv0078 deletion with the WT gene restored iP-control of

276 loss of acid-fast staining (**Figure 6**, panels i and j) but complementation with
277 $Rv0078_{W100R}$, which could not fully de-repress Rv0077c expression in the presence of iP
278 (**Figure. 2C**), could not restore iP-induced loss of acid-fast staining (**Figure 6**, panels k
279 and l). Mixing and simultaneous staining of the Rv0077c and Rv0078 mutants further
280 showed the staining differences were not a result of a technical artifact (**Figure 6,** panel
281 m).

282 Acid-fast staining is primarily thought to be associated with mycolic acids;
283 alterations in mycolic acid synthesis result in negative effects on cell growth *in vitro* and
284 *in vivo*(Barkan *et al.*, 2009, Bhatt *et al.*, 2007). We used thin layer and liquid or gas
285 chromatography coupled to mass spectrometry to analyze the fatty acid, mycolic acid
286 and lipid contents of the WT, Rv0077c and Rv0078 strains and observed no significant
287 qualitative or quantitative differences between strains (**Figure supplement 4, table**
288 **supplement 6**). In particular, the expression of Rv0077c did not conspicuously modify
289 the chain length of mycolic acids, their cyclopropanation or the relative abundance of
290 keto- to alpha- mycolates, and did not alter the wax ester and triglyceride content of the
291 strains that have been previously been linked to acid-fast staining (Bhatt *et al.*, 2007,
292 Deb *et al.*, 2009).

293

294 **Discussion**

295 Our studies are the first to demonstrate CKs induce robust and specific transcriptional
296 and physiologic changes in a bacterial species. *M. tuberculosis* treated with CK
297 expressed high levels of Rv0077c, which altered its metabolome and staining
298 properties. Despite these changes, we did not observe an effect on the susceptibility of

299 these bacteria to antibiotics of different classes or on survival in mice. In addition we
300 found the transcriptional regulator Rv0078 represses Rv0077c expression in the
301 absence of CK, and we defined the operator to which Rv0078 binds. We determined
302 that two dimers of Rv0078 bind to inverted repeats in the intergenic region between
303 Rv0077c and Rv0078, which likely represses the expression of both genes.

304 In plants, CKs are sensed by membrane receptors related to the sensors of
305 bacterial two-component systems (TCS) (To & Kieber, 2008, Inoue *et al.*, 2001, Steklov
306 *et al.*, 2013). In these systems, CKs interact with membrane receptors with a CHASE
307 (cyclases/histidine kinases associated sensing extracellular) domain (Mougel & Zhulin,
308 2001, Anantharaman & Aravind, 2001). As in bacteria, the ligand-receptor interaction
309 stimulates a phospho-relay that ultimately leads to the phosphorylation of a response
310 regulator protein, which either represses or activates gene expression. *M. tuberculosis*
311 has at least 12 known TCS, and none has a predicted CHASE domain (L. Aravind,
312 February 2012; and I. Jouline, August 2017, personal communications). Thus, it
313 remains to be determined if a TCS sensor protein is involved in CK signal transduction
314 in *M. tuberculosis*.

315 It also remains to be determined what the natural ligand is for Rv0078; while
316 SMARt-420 binds robustly to Rv0078 (Wohlkonig *et al.*, 2017, Blondiaux *et al.*, 2017)
317 the cytokinin iP did not in our studies. This result may not be surprising when
318 considering SMARt-420 and cytokinins bear no resemblance to each other.
319 Furthermore, we could not co-crystalize iP with Rv0078 (data not shown). We
320 hypothesize that the interaction of CK with an unknown receptor or enzyme leads to the
321 synthesis of a small molecule (e.g., a lipid) that binds to Rv0078, resulting in the

322 derepression of Rv0077c. This possibility may be supported by our observation that
323 there is an increase in several phospholipids in iP-treated *M. tuberculosis*. We are
324 currently working to identify factors required for CK-mediated gene induction.

325 Rv0077c is predicted to have an α/β hydrolase fold (Soding *et al.*, 2005), which
326 can have a variety of substrate types (Nardini & Dijkstra, 1999). Based on our
327 metabolomics and microscopy studies, we predict Rv0077c targets one or more
328 components of the cell envelope. The identification of the natural target of Rv0077c may
329 help provide new insight into the elusive molecular basis of acid-fastness of this human-
330 exclusive pathogen. Our results may also indicate that acid fast staining can be affected
331 by different cell envelope chemistries in addition to changes in mycolic acid structure or
332 content.

333 While we did not observe any differences in bacterial burden in mice infected
334 with strains that either were disrupted for or constitutively expressed Rv0077c, it is
335 possible that Rv0077c function might only be needed during very late or specific stages
336 of infection. This idea may be supported by a previous observation that the cytokinin
337 synthase *log* gene in *M. marinum* is specifically expressed in late granulomas of
338 infected frogs (Ramakrishnan *et al.*, 2000). Furthermore, mice might not provide an
339 optimal model to observe a role for this pathway in tuberculosis.

340 A previous report suggested the expression of Rv0077c increases the sensitivity
341 of *M. tuberculosis* to the antibiotic ETH. ETH must be activated by a mono-oxygenase,
342 EthA, in order for it to be toxic to *M. tuberculosis* (DeBarber *et al.*, 2000, Vannelli *et al.*,
343 2002). We found the expression of Rv0077c did not confer increased susceptibility to
344 ETH or any other antibiotic we tested, an observation consistent with the likelihood that

345 Rv0077c is not a mono-oxygenase. It is possible that the effects of SMARt-402 is
346 growth-condition dependent, that this molecule affects another pathway to either
347 increase the susceptibility of *M. tuberculosis* to ETH, or that another SMARt-420-
348 induced enzyme in addition to Rv0077c synergize together to activate ETH (A. Baulard,
349 personal communication). Irrespective of these possibilities, it is unlikely that Rv0077c
350 has a considerable role in the activation of ETH. While a previous report named
351 Rv0077c and Rv0078, EthA2 and EthR2, respectively, we propose to rename them
352 "LoaA" and "LoaR" for "loss of acid-fast staining A and Repressor" due to the lack of
353 association of these proteins with ETH susceptibility.

354 Finally, our studies have opened the door to the possibility that numerous
355 commensal and pathogenic microbes (including fungi) use cytokinins for intra- or inter-
356 species communication in complex systems such as the gut microbiome. The
357 identification of one or more CK receptors and the signal transduction pathway that
358 leads to the induction of Rv0077c expression will likely lay the foundation for
359 understanding CK signaling in hundreds of bacterial species.

360

361 **Author Contributions** M.I.S. and K.H.D. performed *in vitro* and *in vivo M. tuberculosis*
362 work. H.C.H. and H.L. determined the structure of Rv0078 and performed the
363 mutagenesis and EMSA assays. M.B.J. performed the RNA-Seq analysis. S.H.B. and
364 A.T.J. performed antibiotic susceptibility assays. M.S. provided cytokinins and their
365 ribosides. V.J., M.R.M. and M.J. performed the lipid analysis. C.X. performed mass
366 spectrometry of fatty acids extracted from purified Rv0078 protein. M.S. synthesized
367 cytokinins and their precursors. M.I.S., H.C.H., H.L. and K.H.D. wrote the manuscript.

368 **Acknowledgments** We thank A. Darwin and V. Torres for critical review of a draft
369 version of this manuscript. We thank S. Zhang for assistant in preparing reagents. This
370 work was supported by NIH grant R01 HL092774 awarded to K.H.D. K.H.D. holds an
371 Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs
372 Wellcome Fund. M.I.S. and S.H.B were supported by the Jan T. Vilcek Endowed
373 Fellowship fund. S.H.B. is also supported by NIH grant T32 AT007180. H.C.H. and H.L.
374 were supported by R01 AI070285. M. Strnad was supported by LO1204 from the
375 National Program of Sustainability I. We thank A. Liang and Y. Deng of the Microscopy
376 Laboratory at New York University Langone Medical Center for assistance with
377 microscopy and imaging. Diffraction data for this study were collected at the Lilly
378 Research Laboratories Collaborative Access Team (LRL-CAT) beamline and the Life
379 Sciences Collaborative Access Team (LS-CAT) beamline at the Advanced Photon
380 Source (APS), Argonne National Laboratory. APS was supported by the U.S.
381 Department of Energy, Office of Science, Office of Basic Energy Sciences, under
382 Contract DE-AC02-06CH11357. Use of the LRL-CAT beamline at Sector 31 of the APS
383 was provided by Eli Lilly Co., which operates the facility.
384

385 **Materials and Methods**

386 **Bacterial strains, plasmids, primers, chemicals, and culture conditions.** Bacterial
387 strains, plasmids, and primer sequences used in this study are listed in Table
388 supplement 1. All primers for cloning and sequencing were from Invitrogen, Inc. *M.*
389 *tuberculosis* strains were grown in Middlebrook 7H9 broth (Difco) supplemented with
390 0.2% glycerol, 0.05% Tween-80, 0.5% fraction V bovine serum albumin, 0.2% dextrose

391 and 0.085% sodium chloride ("7H9c"). *M. tuberculosis* cultures were grown without
392 shaking in 25 or 75 cm^2 vented flasks (Corning) at 37 ºC. 7H11 agar (Difco)
393 supplemented with 0.5% glycerol and BBL TM Middlebrook OADC enrichment (BD) was
394 used for growth on solid medium ("7H11"). *M. tuberculosis* was transformed as
395 described(Hatfull & Jacobs, 2000). *E. coli* strains used for cloning and expression were
396 grown in LB-Miller broth (Difco) at 37 °C with aeration on a shaker or on LB agar. *E. coli*
397 strains were chemically transformed as previously described(Sambrook *et al.*, 1989).
398 The final concentrations of antibiotics used for *M. tuberculosis* growth: kanamycin, 50
399 µg/ml; hygromycin, 50 µg/ml; streptomycin, 25 µg/ml; and for *E. coli*: hygromycin, 150
400 µg/ml; kanamycin, 100 µg/ml; and streptomycin 50 µg/ml. AMP, adenine and iP were
401 purchased from Sigma. iPR, 2MeSiP and 2MeSiPR were synthesized as previously
402 described(Sugiyama & Hashizume, 1978). The purity of the synthesized cytokinin and
403 derivatives was >98% for each as determined by high performance liquid
404 chromatography and mass spectrometry.

405 The Rv0077c::MycoMarT7 mutant was isolated from a library of ordered
406 transposon insertion mutants as previously described(Darwin *et al.*, 2003, Festa *et al.*,
407 2007). The ΔRv0078::*hyg* mutant was made by deletion-disruption mutagenesis as
408 described in detail elsewhere using pYUB854(Festa *et al.*, 2011, Bardarov *et al.*, 2002).
409

410 **Protein purification and immunoblotting.** DNA sequence encompassing the full-
411 length Rv0078 or Rv0077c gene was cloned into pET24b(+) vector using primers listed
412 in Table supplement 1. Recombinant proteins were produced in *E. coli* ER2566 and
413 purified under native conditions for Rv0078 and denaturing conditions for Rv0077c

414 according to the manufacturer's specifications (Qiagen). Polyclonal rabbit antibodies
415 were raised by Covance (Denver, PA). For all immunoblots, cell lysates or purified
416 proteins were separated sodium dodecyl sulfate polyacrylamide gel electrophoresis
417 (SDS-PAGE); transferred to nitrocellulose and incubated with rabbit polyclonal
418 antibodies to the protein of interest at 1:1000 dilution in 3% bovine serum albumin in
419 TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20, pH 7.4). Equal loading
420 was determined by stripping the nitrocellulose membranes with 0.2 N NaOH for 5 min,
421 rinsing, blocking and incubating the nitrocellulose with polyclonal rabbit antibodies to
422 dihydrolipoamide acyltransferase (DlaT)(Tian *et al.*, 2005). Horseradish peroxidase
423 conjugated anti-rabbit antibody (GE-Amersham Biosciences) was used for
424 chemiluminescent detection (SuperSignal West Pico; ThermoScientific).

425 For crystallography studies of Rv0078-His_6, bacteria were grown at 37°C to an
426 OD_{600} = 0.5-0.6 before being induced with 0.5 mM IPTG and incubated at 16°C
427 overnight. After harvesting by centrifugation, cells were lysed by passing through a
428 microfluidizer cell disruptor in 10 mM potassium phosphate, pH 8.0, 10 mM imidazole,
429 and 500 mM NaCl. The homogenate was clarified by spinning at 27,000 *g* and the
430 supernatant was applied to a HiTrap-Ni column (GE Healthcare) pre-equilibrated with
431 the lysis buffer. Histidine-tagged protein was eluted with a 10–300 mM imidazole
432 gradient in 10 mM potassium phosphate, pH 8.0, containing 300 mM M NaCl. The
433 Rv0078 fractions were further purified by a Superdex 75 column (16 x 1000 mm, GE
434 Healthcare) pre-equilibrated with 20 mM potassium phosphate, pH 8.0, and 300 mM
435 NaCl. The purified Rv0078 was concentrated to 40 mg/ml for crystallization screen.
436

437 **RNA-Seq and 5'RACE.** Three biological replicate cultures of WT *M. tuberculosis* were
438 grown to an OD_{580} ~1 and incubated in 100 μM iP or DMSO (control) for five hours.
439 Cells were harvested and RNA was purified as described previously (Festa *et al.*, 2011).
440 Briefly, an equal volume of 4 M guanidinium isothiocyanate, 0.5% sodium N-lauryl
441 sarcosine, 25 mM trisodium citrate solution was added to cultures to arrest transcription.
442 RNA was isolated with Trizol Reagent (Invitrogen) and further purified using RNeasy
443 Miniprep kits and DNase I (Qiagen). Transcriptome profiling by RNA-seq was performed
444 and analyzed as follows: RNA from *M. tuberculosis* cultures were extracted for library
445 construction. Libraries were constructed and barcoded with the Epicentre ScriptSeq
446 Complete Gold low input (Illumina, Inc) and sequenced on Illumina HiSeq 2000
447 sequencer using version 3 reagents. Unique sequence reads were mapped to the
448 corresponding reference genome and RPKM values were calculated in CLC (CLC
449 version 7.0.4). Genes with significantly different RPKM values were identified using the
450 Significant Analysis for Microarray (SAM) statistical analysis component of MeV(Saeed
451 *et al.*, 2003).
452 5'RACE was performed as described by the manufacturer (Invitrogen). Briefly, 1
453 μg of RNA was used as template for cDNA production using a reverse primer 150–300
454 bp downstream of annotated translational start sites. A 3' poly-C tail was added to
455 cDNA by recombinant Tdt. The cDNA was then amplified using a nested reverse primer
456 and a primer that anneals to the poly-C tail. Products were cloned and sequenced.
457 Likely transcriptional start sites were selected based on clones that had the most
458 nucleotide sequence upstream of the start codon.
459

460 **EMSA.** A series of double stranded DNA probes consisting of sequences in the
461 intergenic region between Rv0077c and Rv0078 were generated by annealing two
462 complementary oligonucleotides and a 5'-end IRDye700-labeled 14-nucleotide
463 oligomers (5'dye-GTGCCCTGGTCTGG-3') (Integrated DNA Technologies). Binding
464 assays were performed by incubating 100 nM of probes and various concentrations of
465 Rv0078 at room temperature for 30 min in 20 mM HEPES, pH 7.5, 3 mM DTT, 0.1 mM
466 EDTA, 100 mM KCl, 5% glycerol, 5 mg/ml BSA, 10 mM $MgCl_2$, and 0.25% Tween 20,
467 and were subsequently resolved in 6% polyacrylamide gels in 0.5 × TBE buffer. Mobility
468 shifts of protein-DNA complex were visualized in LI-COR Odyssey imager.
469
470 **Crystallization and structure determination.** DNA-free Rv0078 crystals were
471 obtained by screening at 20 °C using the sitting-drop vapor diffusion method. The C2
472 space group crystals were grown in 0.1 M sodium cacodylate, pH 6.4, and 1.3 M
473 Lithium sulfate. SeMet substituted Rv0078 crystals with C2 space group were grown in
474 in 0.1 M sodium cacodylate, pH 6.6, 1.3 M Lithium sulfate, 0.2 M magnesium sulfate,
475 and 2% PEG400. Diffraction data to a resolution of 1.85 Å were collected at the Lilly
476 Research Laboratories Collaborative Access Team (LRL-CAT) beamline of Advanced
477 Photon Source (APS), Argonne National Laboratory, and were processed with Mosflm
478 software(Winn *et al.*, 2011). The program Hybrid-Substructure-Search in the Phenix
479 package was used to locate the Se sites and the initial phasing was carried out using
480 the program Autosol of Phenix. The 2.7 Å map phased by SAD method allowed us to
481 build Rv0078 model unambiguously. The native Rv0078 structure was subsequently
482 determined by the program PHASER using SeMet substituted Rv0078 as the initial

483 search model. To obtain the Rv0078-DNA crystals, purified Rv0078 was co-crystallized
484 with a 23-mer DNA duplex (5'- TTTACAAGCAGACTGCCGGTAAC-3') at a molar ratio
485 of 2:1 (protein dimer:DNA) in the presence of 150 mM $MgCl_2$. The DNA-bound Rv0078
486 crystals were grown in the buffer containing only 0.2 M Magnesium formate. Diffraction
487 data to 3.0 Å were collected at the Life Sciences Collaborative Access Team (LS-CAT)
488 beamline of APS and were processed with Mosflm. The Rv0078-DNA structure was
489 determined by PHASER using DNA-free structure as the search model. All the
490 refinements were performed using Phenix-refine (Adams *et al.*, 2010). The statistics
491 were provided in **Supplemental Table 3**.
492
493 **Mass spectrometry of fatty acids co-purified with Rv0078.** Fatty acids were
494 extracted from 5 mg Rv0078 that was purified from *E. coli*, and analyzed as described
495 previously (Fan *et al.*, 2013). Separation and identification of the FA methyl esters were
496 performed on an HP5975 gas chromatograph-mass spectrometer (Hewlett-Packard)
497 fitted with a 60 m × 250 μm SP-2340 capillary column (Supelco) with helium as the
498 carrier gas
499
500 **Isothermal calorimetry.** ITC experiment was performed in a Microcal PEAQ-ITC at 25
501 °C. The stirring speed was 750 rpm and the interval between each titration was 150 sec.
502 The concentration of Rv0078 in the reaction cell was 25 μM and the concentration of the
503 titration DNA ligand was 500 μM. The recorded thermal data was analyzed using
504 Microcal PEAQ-ITC analysis software.
505

506 **MIC_{50} Determination.** To determine the MIC_{50} of each antibiotic, *M. tuberculosis* strains
507 were grown to an OD_{580} ~0.7 and diluted into fresh media to an OD_{580} of 0.02. Diluted
508 cultures were transferred to a 96-well microtiter plate containing triplicate, 10-fold serial
509 dilutions of antibiotic. Cell wall-active antibiotics (vancomycin, meropenem) were
510 supplemented at all concentrations with potassium clavalunate to inhibit the intrinsic β-
511 lactamase activity of *M. tuberculosis*. After five days of incubation at 37ºC, growth of
512 each strain was measured by OD_{580}. MIC_{50} values were interpolated from a non-linear
513 least squares fit of log_2-transformed OD_{580} measurements. Data are representative of
514 two independent experiments. Antibiotics were purchased from Sigma-Aldrich
515 (clavulanate, ethionamide, meropenem, rifampicin, vancomycin) or Thermo-Fisher
516 Scientific (ciprofloxacin, ethambutol, isoniazid, norfloxacin, streptomycin).
517
518 **Mouse Infections.** Mouse infections were performed essentially as described
519 previously(Darwin *et al.*, 2003). 7-9-week-old female C57BL6/J mice (The Jackson
520 Laboratory) were infected by aerosol to deliver ~200 bacilli per mouse, using a Glas-Col
521 Inhalation Exposure System (Terre Haute, IN). Strains used were WT (MHD761) or
522 (MHD794), Rv0077c (MHD1086), Rv0077c complemented (MHD1077), Rv0078
523 (MHD1315), Rv0078 complemented with WT Rv0078 (MHD1318) and $Rv0078_{W100R}$
524 (MHD1316). This study was performed in strict accordance with the recommendations
525 in the Guide for the Care and Use of Laboratory Animals of the National Institutes for
526 Health. Mice were humanely euthanized according to an approved Institutional Animal
527 Care and Use Committee protocol at New York University School of Medicine. Lungs

528 and spleens were harvested and homogenized PBS/0.05% Tween-80 at indicated time
529 points to determine bacterial CFU.
530
531 **Metabolomic analysis of *M. tuberculosis* cell lysates.** Four independent cultures of
532 each analyzed strain were grown in 7H9 to an OD_{580} ~0.7 and treated with iP in DMSO
533 at a final concentration of 100 μM or an equal volume of DMSO for 24 hours. Bacteria
534 were harvested the next day at OD_{580} ~1. Sixty-five OD-equivalents per replicate were
535 processed by chloroform:methanol extraction(Layre *et al.*, 2011, Samanovic *et al.*,
536 2015). Metabolomic profiling was performed by Metabolon, Inc.
537
538 **Staining and microscopy.** *M. tuberculosis* strains were grown to mid-logarithmic
539 phase (OD_{580} ~0.5-0.7). 5 μl of culture was spotted onto glass slides and heat-killed
540 over a flame or on a heat block (15 min, 80˚C). Staining was performed according the
541 method of Ziehl-Neelson as per the manufacturer's instructions (BD Stain Kit ZN).
542 Images were acquired on a Zeiss Axio Observer with a Plan-Aprochromat 63x/1.4 oil
543 lens. Images were taken with an Axiocam503 camera at the NYULMC Microscopy
544 Laboratory.
545
546 **Analysis of total lipids, mycolic acids and shorter chain fatty acids.** For lipid
547 analysis 400 ml cultures were grown up to OD_{580} ~0.7 and treated with iP in DMSO at a
548 final concentration of 100 μM or an equal volume of DMSO-only for 24 hours. 400 ml of
549 Rv0077c and Rv0078 cultures were treated with DMSO only. Cells were washed three
550 times in DPBS and heated at 100°C for 45 min for sterilization before freezing at -20 °C.

551 Total lipids extraction from bacterial cells and preparation of fatty acid and mycolic acid
552 methyl esters from extractable lipids and delipidated cells followed earlier procedures
553 (Stadthagen *et al.*, 2005). Total lipids and fatty acid/mycolic acid methyl esters were
554 analyzed by one and two-dimensional thin-layer chromatography (TLC) in a variety of
555 solvent systems on aluminum-backed silica gel 60-precoated plates F_{254} (E. Merck).
556 TLC plates were revealed by spraying with cupric sulfate (10% in a 8% phosphoric acid
557 solution) and heating. Alternatively, total lipids were run in both positive and negative
558 mode and the released fatty acids/mycolic acids in negative mode only, on a high
559 resolution Agilent 6220 TOF mass spectrometer interfaced to a LC as described(Sartain
560 *et al.*, 2011, Bhamidi *et al.*, 2012). Data files were analyzed with Agilent's Mass hunter
561 work station software and most compounds were identified using a database of *M.*
562 *tuberculosis* lipids developed in-house (Sartain *et al.*, 2011). Fatty acids methyl esters
563 from extractable lipids were treated with 3 M HCl in CH_3OH (Supelco) overnight at 80˚C,
564 dried and dissolved in *n*-hexane(s) prior to GC/MS analysis. GC/MS analyses of fatty
565 acid methyl esters were carried out using a TRACE 1310 gas chromatograph (Thermo
566 Fisher) equipped with a TSQ 8000 Evo Triple Quadrupole in the electron impact mode
567 and scanning from *m/z* 70 to *m/z* 1000 over 0.8 s. Helium was used as the carrier gas
568 with a flow rate of 1 ml per min. The samples were run on a ZB-5HT column (15 m x
569 0.25 mm i.d.) (Zebron). The injector (splitless mode) was set for 300°C (350°C for
570 mycolic acid methyl esters). The oven temperature was held at 60°C for 2 min,
571 programmed at 20°C per min to 375°C, followed by a 10 min hold. The data analyses
572 were carried out on Chromeleon data station.
573

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719

Samanovic, Hsu et al, Figure 1

A

Gene	Putative function	Fold increase
Rv0076c	Hypothetical protein	3
Rv0077c	Oxidoreductase	81
Rv0078	TetR-like regulator	2
Rv1557	*mmpL6*; possible lipid transporter	8

B

Gene	*Mma*	*Mav*	*Mul*	*Mle*	*Mgi*	*Msm*
Rv0076c	76	68	0	0	0	58
Rv0077c	87	88	86	0	53	52
Rv0078	94	90	92	67	0	0
*mmpL6**	88	88	76	63	80	83

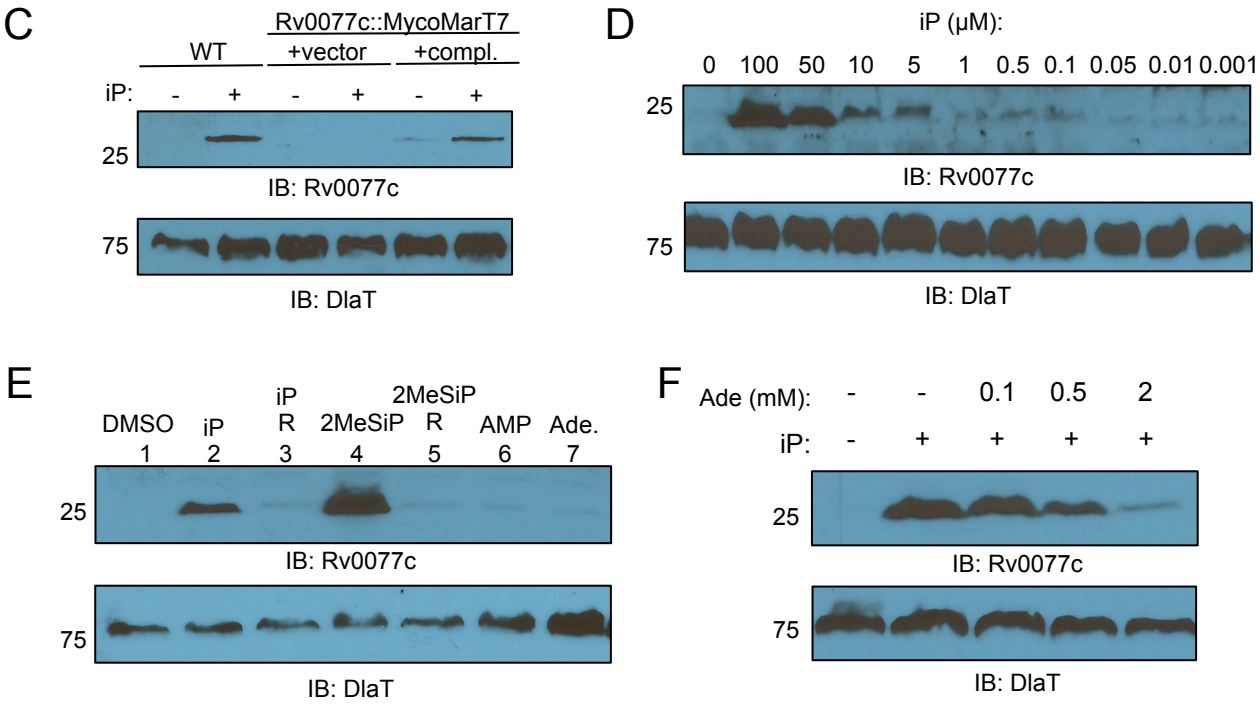

Figure 1. Cytokinins induce the expression of Rv0077c in *M. tuberculosis*. (A) Genes significantly regulated by the presence of 100 μM of iP for five hours as analyzed by RNA-Seq. **(B)** Percent identity between *M. tuberculosis* H37Rv proteins and proteins of targeted mycobacterial genomes including *M. marinum* (*Mma*), *M. avium* (*Mav*), *M. ulcerans* (*Mul*), *M. gilvum* (*Mgi*), and *M. smegmatis* (*Msm*). Asterisk (*) indicates *mmpL6* encodes a truncated protein in H37Rv, unlike in the other mycobacterial species in the table. **(C)** Immunoblot for Rv0077c in total cell lysates of WT *M. tuberculosis*. "compl." = complemented. iP was used at a final concentration of 100 μM when added. **(D)** Dose-dependent production of Rv0077c protein. Bacteria were incubated with cytokinin at the indicated concentrations for 24 hours. **(E)** Only cytokinins, and not closely related molecules, induce the production of Rv0077c. Each compound was added to a final concentration of 100 μM. "R" indicates the riboside form of the preceding indicated cytokinin. **(F)** Adenine inhibits the induction of Rv0077c by 100 μM iP. For all panels, we added an equal volume of DMSO to samples where iP was not added. For all immunoblots (IB), we stripped the membranes and incubated them with antibodies to dihydrolipoamide acyltransferase (DlaT) to confirm equal loading of total lysates. Molecular weight standards are indicated to the left of the blots and are in kilodaltons (kD). Ade, adenine.

Samanovic, Hsu et al, Figure 2

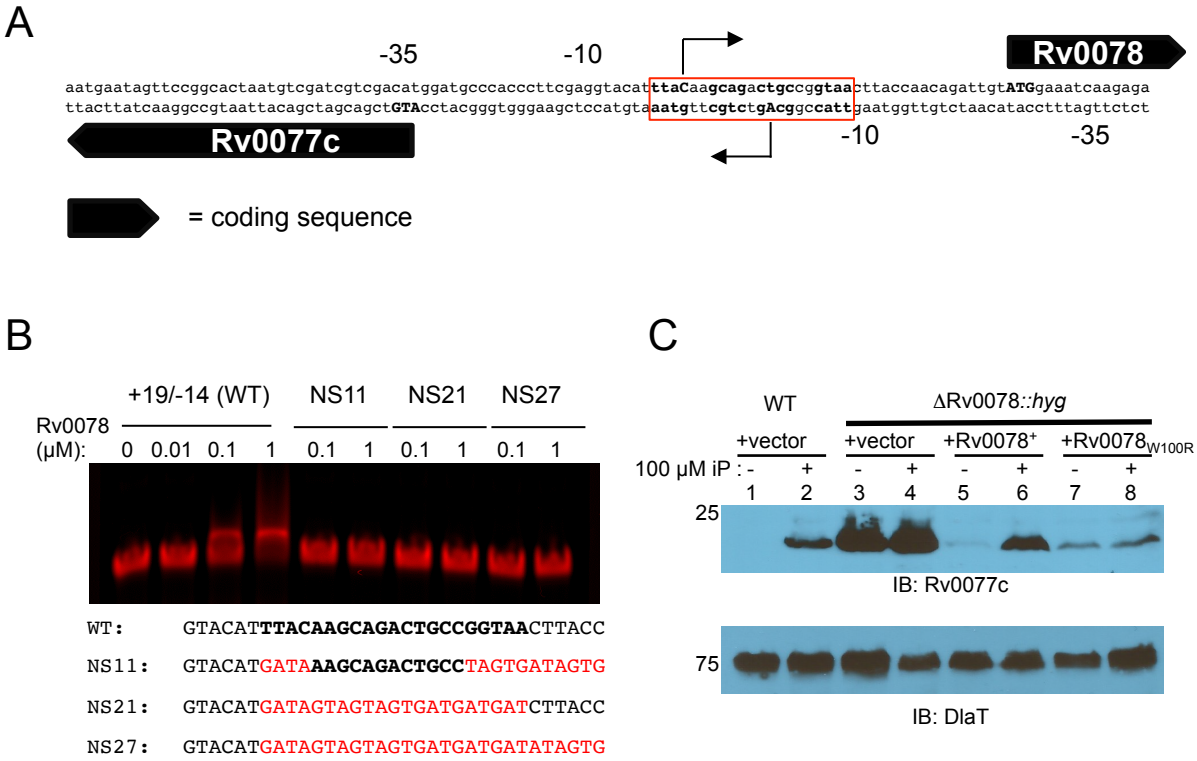

Figure 2. Rv0078 represses the expression of Rv0077c. (A) The putative transcriptional start sites (+1) of Rv0077c and Rv0078 as determined by 5'RACE and represented as bent arrows. The predicted start codons are in capital bold letters. **(B)** EMSA analysis identifies a putative repressor binding site. Probe sequences. +19/-14 refers to positions relative to the Rv0077c +1. In bold is the presumed binding site. Mutated residues are in red. Not shown at the end of each probe is a sequence for annealing to a fluorescent tag (Table supplement 1). Rv0078 was purified under native conditions from *E. coli*. **(C)** Deletion and disruption of Rv0078 results in the constitutive expression of Rv0077c. Total cell lysates were prepared and separated on a 10% SDS-PAGE gel. IB = immunoblot. The membrane was stripped and incubated them with antibodies to DlaT to confirm equal loading of samples. Molecular weight standards are indicated to the left of the blots and are in kilodaltons (kD).

Samanovic, Hsu et al, Figure 3

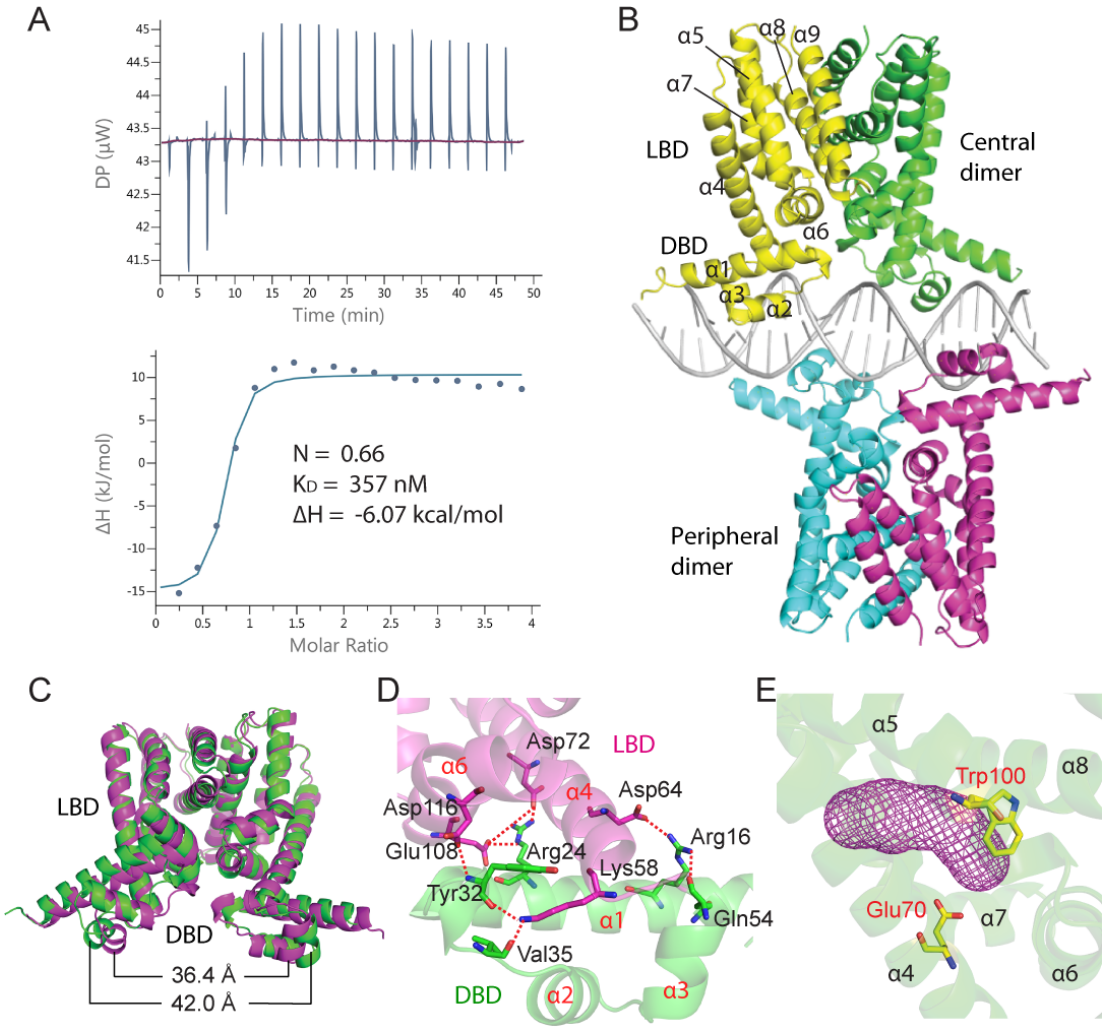

Figure 3. Crystal structure of Rv0078 in complex with DNA. (A) ITC of Rv0078 binding to the +13/-8 DNA probe. The binding stoichiometry, ΔH, and K_D are marked. **(B)** Overall structure of Rv0078-DNA complex in cartoon view. Two Rv0078 dimers ("central" and "peripheral") bind to one DNA molecule. **(C)** The distance between two DNA-binding domains decreases by ~6 Å when bound to DNA. The DNA-free Rv0078 is in green; the DNA-bound Rv0078 is in magenta. **(D)** Interactions between the LBD (magenta) and DBD (green) in a monomer. **(E)** The ligand binding pocket (magenta mesh) of Rv0078 is enclosed by a four-helix bundle (helices α5 to α8).

Samanovic, Hsu et al, Figure 4

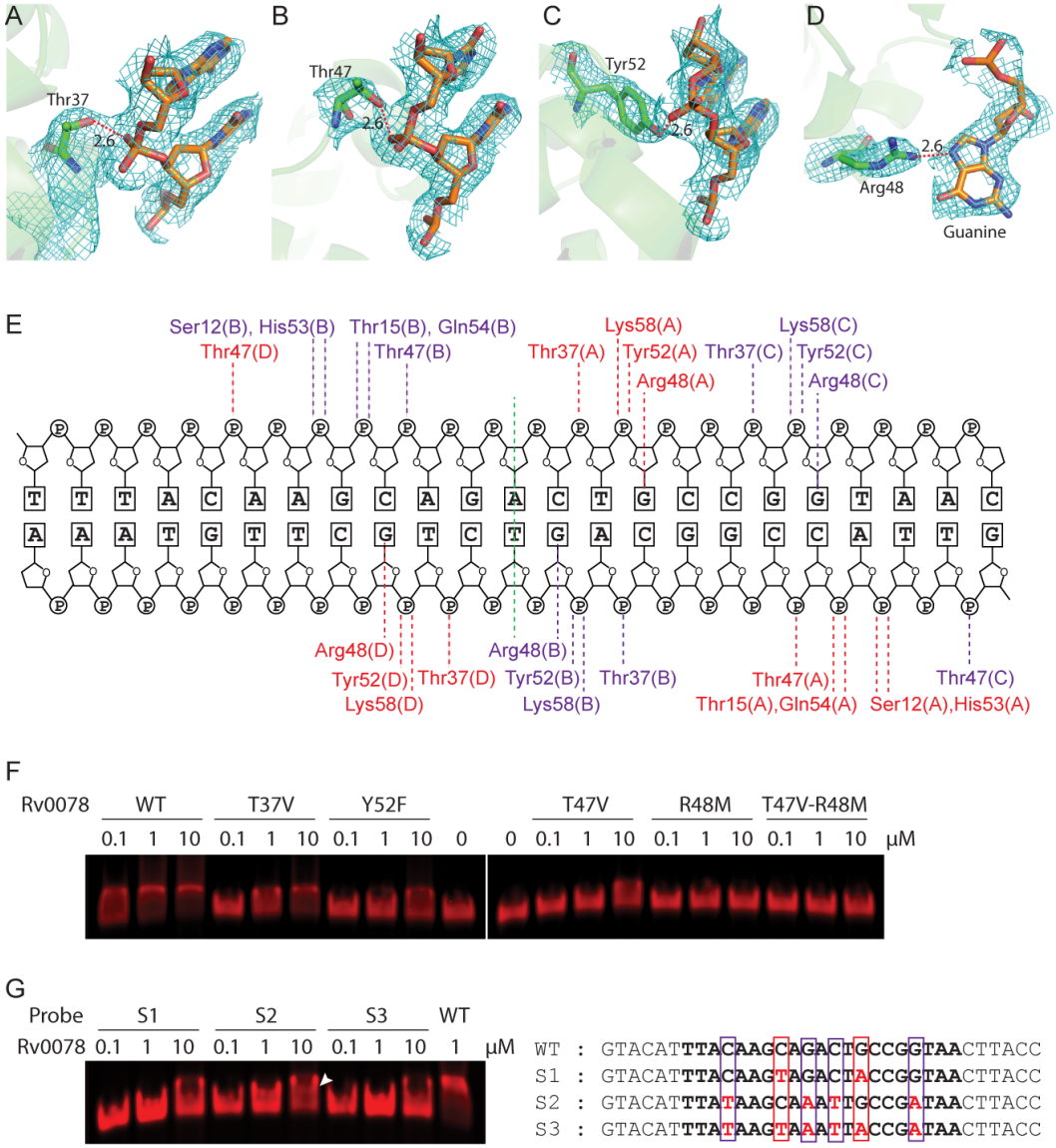

Figure 4. Rv0078-DNA interactions. The hydroxyl group of **(A)** Thr37, **(B)** Thr47, and **(C)** Tyr52 interact with the backbone phosphate with a distance of 2.6 Å. **(D)** Arg48 interacts with guanidine with a distance of 2.6 Å. The 2Fo-Fc maps are contoured at 1σ level. **(E)** A schematic representation of Rv0078-DNA contacts. Residues of the central dimer are labeled in red, and residues of the peripheral dimer are in purple. **(F)** EMSA using WT DNA probe and Rv0078 with mutations in Thr37, Thr47, Arg48, or Tyr52. **(G)** Left panel: EMSA of Rv0078 with DNA probes with G-to-A substitutions in the central dimer binding region (S1), peripheral dimer binding region (S2), or in both DNA regions (S3). The white arrowhead marks the partial shift with the S2 probe at high protein concentration. Right panel: sequences of the four DNA probes used in EMSA experiments. The nucleotides contacting the central dimer are boxed in red and those contacting the peripheral dimer are boxed in purple.

Samanovic, Hsu et al, Figure 5

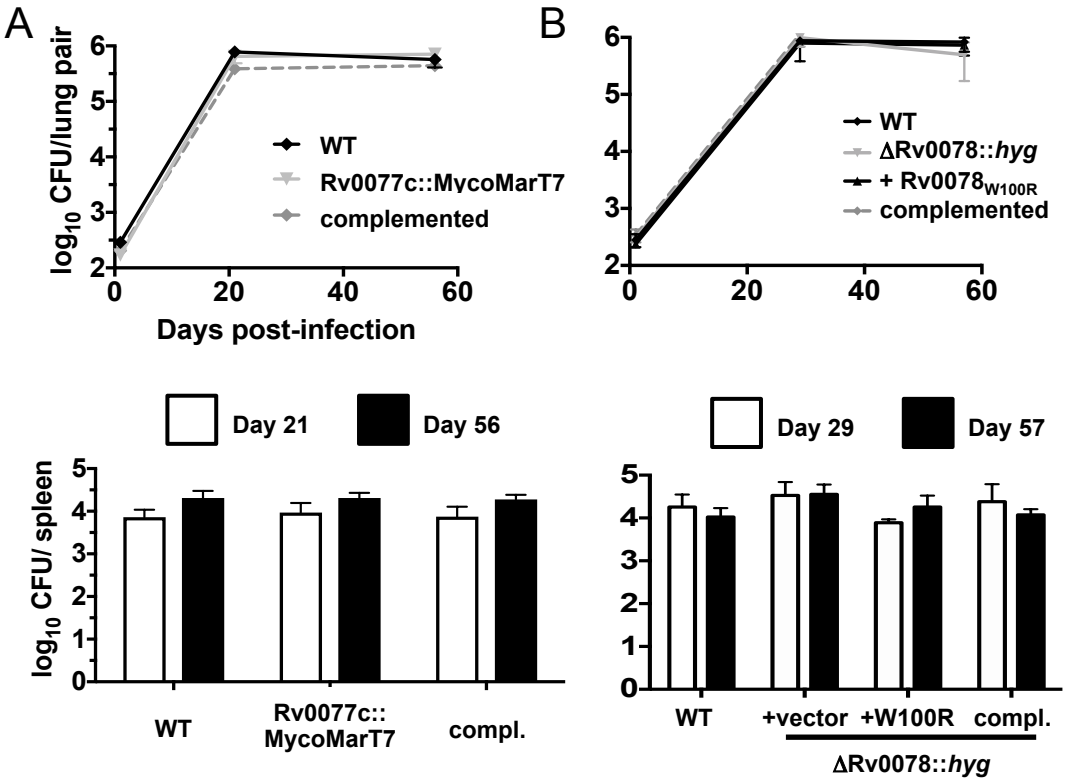

Figure 5. Loss of Rv0077c or Rv0078 does not attenuate bacterial survival in mice. **(A)** Bacterial colony forming units (CFU) after infection of C57BL/6J mice with WT, Rv0077c and complemented strains. **(B)** Bacterial CFU after infection of C57BL/6J mice with WT, Rv0078 and complemented strains. For both panels, data in each are from a single experiment that is representative of two independent experiments. Error bars indicate the standard error of the mean.

Samanovic, Hsu, et al, Figure 6

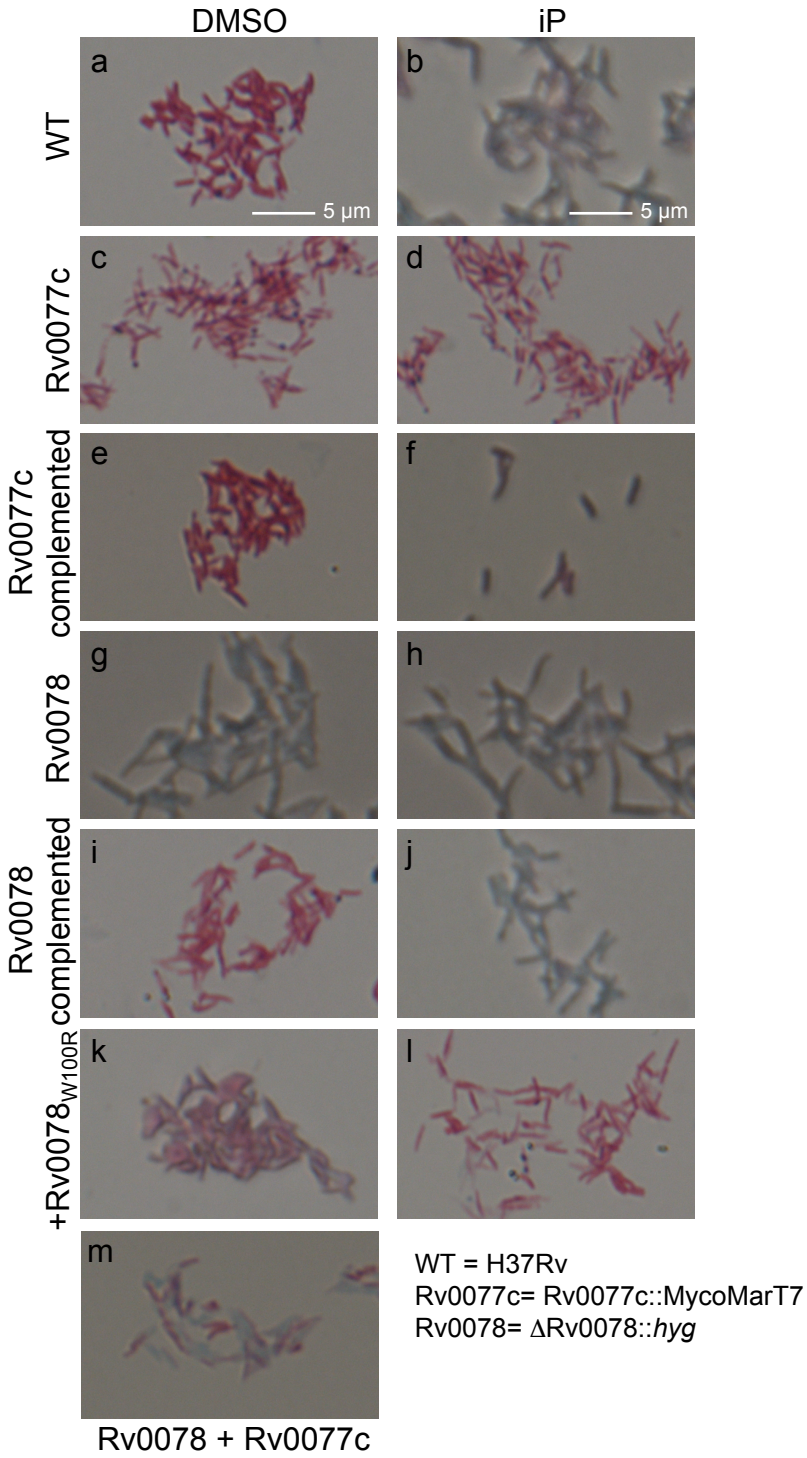

Figure 6. Induction of Rv0077c expression results in loss of acid-fast staining. *M. tuberculosis* strains were examined by Ziehl-Neelsen staining. Magnification is 63-fold. Scale bars are applicable to all images. See text for details.