Mycobacteria recycle their peptidoglycan *via* a novel pathway which influences antimicrobial resistance and limits proliferation in macrophages.

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Abstract

Growth and division by most bacteria requires remodeling and cleavage of their cell wall. A byproduct of this process is the generation of free peptidoglycan (PG) fragments known as muropeptides. These muropeptides are recycled in many model organisms, where the bacteria can harness their unique nature as a signal for cell wall damage. These molecules also serve as important signals for hosts where binding to specific receptors reports on the presence of intracellular bacteria. Despite this critical role for muropeptides, it has long been thought that pathogenic mycobacteria such as *Mycobacterium tuberculosis* do not recycle their PG. Herein we show that *M. tuberculosis* and *Mycobacterium bovis* BCG are both able to recycle components of their PG. We demonstrate that Mur*N*Ac but not Glc*N*Ac can be metabolised by

40 mycobacteria and that stem-peptide recycling proceeds independent of amino sugar recovery.
41 In addition, we demonstrate that the core-mycobacterial gene *lpqI* encodes an authentic NagZ
42 β-*N*-acetylglucosaminidase, which is essential for recycling Mur*N*Ac. Surprisingly, loss of *lpqI*
43 leads to antimicrobial resistance and increased proliferation in macrophages. This supports a
44 model whereby the amount of PG released by mycobacterial cells is tightly controlled in order
45 to effectively modulate the infection process.

46 Introduction

47 For most bacteria maintenance of a peptidoglycan (PG) sacculus is an essential aspect
48 of life. PG is a heteropolymer comprised of glycan chains with a repeating disaccharide motif
49 of *N*-acetylglucosamine β1→4 *N*-acetylmuramic acid (Glc*N*Ac-Mur*N*Ac) which are then cross-
50 linked to one another *via* short peptides attached to the C-3 D-lactyl moiety of Mur*N*Ac. The
51 integrity of this macromolecule must be maintained under most growth conditions and its
52 rupture leads to lysis and cell death[1]. As a result of this essentiality, it is vital that cells are able
53 to withstand their own internal turgor pressure and still be able to cleave the cell wall to allow
54 for division, growth and the insertion of macromolecular structures such as secretion systems[1].
55 Throughout this process, the activity of lytic enzymes or through the attack of host agents like
56 lysozyme, the sacculus is cleaved with the resulting generation of small PG metabolites known
57 as muropeptides[2].

58 In Gram-positive bacteria muropeptides are typically released from the cell wall through
59 the action of lysozyme-like hydrolytic enzymes, whereas in Gram-negative bacteria, lytic
60 transglycosylases generate 1,6-anhydroMur*N*Ac products[3,4]. These metabolites have been
61 shown to be important in many aspects of host-pathogen interactions. For example, tracheal
62 cytotoxin produced by *Bordetella pertussis* is the product of lytic transglycosylases[5]. Release
63 of a similar molecule has also been shown to be involved in tissue damage during *Neisseria*
64 *gonorrhoeae* infection and in the closure of the light-organ of the bobtail squid[6,7]. In many

65 organisms, soluble PG acts as a potent immune stimulator once sensed by NOD receptors and
66 other pattern recognition receptors[8].

67 Aside from host organisms, PG metabolites are also important signaling molecules for
68 the bacteria themselves. For example, recycling of PG has been studied in great detail in a small
69 number of organisms including *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus*
70 *subtilis* among others[9]. The recycling pathway typically involves the step-wise degradation of
71 the polymer into its monomeric constituents, monosaccharides and amino acids (Figure 1).
72 Despite common biochemical steps, compartmentalization of these steps tends to be organism
73 specific[3]. The resulting monosaccharides are eventually phosphorylated and MurNAc-6-
74 phosphate is converted into glucosamine-6-phosphate through the activities of cytoplasmic
75 MurQ and NagA enzymes (Figure 1). At the same time, the stem peptides are degraded to
76 smaller components and typically shunted back into PG biogenesis. As a result of its presence
77 in many model organisms and pathogens, MurQ-deficient PG recycling has so far only been
78 described in *Pseudomonas putida*, and many bacteria, including mycobacteria, are not thought
79 to recycle their PG at all[10].

80 The cell wall of *M. tuberculosis* is built upon a foundation of PG. The remainder of this
81 structure is formed by the modification of muramic acid residues with an arabinogalactan
82 polymer that is in turn esterified by mycolic acids[11]. This waxy coating contributes to drug
83 resistance in *M. tuberculosis*, but is also the target of several mycobacteria-specific
84 antibiotics[11]. The challenge of multi- and extensively-drug resistant *M. tuberculosis* has not
85 adequately been met by drug discovery efforts, however recent reports suggest that β-lactams
86 are effective at treating these drug-resistant infections[12-14]. Despite their therapeutic promise,
87 we know relatively little about the turn-over of PG in mycobacteria, which is the eventual target
88 of β-lactam antibiotics.

89 In the present study we sought to determine if mycobacteria are capable of recycling
90 their PG and if so, what impact this has on the pathogenicity and immunogenicity of these
91 organisms. We for the first time reveal that these bacteria do indeed possess the biochemical
92 capacity to recycle PG elements and determined the molecular basis of Mur*N*Ac recovery. Our
93 data indicate that loss of a key recycling enzyme, LpqI, leads to increased antibiotic and
94 lysozyme resistance. Surprisingly, loss of this enzyme also leads to increased proliferation in
95 murine bone-marrow-derived macrophages suggesting that PG-recycling plays an important
96 role in maintaining balanced growth in the host.

97 Materials and methods

98 **Bacterial strains and growth conditions:**

99 *M. bovis* BCG (Pasteur) and related mutants were maintained on Middlebrook 7H10
100 agar or 7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween 80. Where
101 appropriate kanamycin or hygromycin was added at 25 or 50 $\mu g \cdot mL^{-1}$, respectively. *M.*
102 *smegmatis* mc^2155 were maintained on Tryptic Soy Broth or Tryptic Soy Agar where
103 appropriate. For growth on defined carbon sources, strains were grown in Sauton's minimal
104 medium (4 g asparagine, 2 g citric acid, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$ * 7 H_2O and 0.05 g ferric
105 ammonium citrate, 0.05% tyloxopol) containing either 5 mM of each sole-carbon unless stated
106 otherwise[15]. *Escherichia coli* strains were grown in lysogeny broth and supplemented with
107 kanamycin at 50 $\mu g \cdot mL^{-1}$ or hygromycin at 150 $\mu g \cdot mL^{-1}$ where appropriate.

108 **Mutant generation**

109 To generate the Δ*lpqI* strain we used specialized transduction according to established
110 protocols[16]. A recombinant *lpqI* knockout phage was designed to replace the chromosomal *lpqI*
111 gene using homologous flanking regions to *lpqI* with a the *sacB* gene and a hygromycin
112 resistance cassette in-between using the LL-Rv0237, LR-Rv0237, RR-Rv0237 and RL-Rv0237

113 primers (all primers are found in Table S2). The resulting phage was transduced into *M. bovis*
114 BCG and transductants were selected on 7H10-agar plates containing 75 μg•mL^{-1} hygromycin.
115 The mutant was verified by PCR and phenotypically with 4MU-Glc*N*Ac where loss of *lpqI* was
116 expected to abolish turn-over of this fluorescent substrate. The complemented strain was
117 generated by incorporating the *lpqI* gene and 150 bp 5' to the start codon containing the
118 promoter sequence into the promoter-less integrative mycobacterial shuttle vector pMV306
119 using primers Rv0237CompF and Rv0237CompR to generate the resulting pMV306-*lpqI*
120 plasmid[17]. This was electroporated into *M. bovis* BCG Δ*lpqI* and transformants were selected
121 on 7H10 agar containing hygromycin and kanamycin. The complemented mutant was verified
122 phenotypically with 4MU-Glc*N*Ac. A similar strain was constructed using the empty pMV306
123 vector.

124 **Antimicrobial testing**

125 Mid-exponential cultures of *M. bovis* BCG and derivative strains were diluted to the
126 indicated OD_{600} in fresh 7H9 media. 100 μL of this culture was added to a 96-well plate with
127 the addition of 1 μL of antibiotic/lysozyme to achieve the desired final concentration as
128 indicated. These were incubated for 7 days at 37 °C at which point 30 μL of 0.02% w/v resazurin
129 and 12.5 μL of 20% Tween 80 v/v was added to the culture. This was incubated over-night at
130 37 °C and the production of resorufin was determined by fluorescence (Ex. 530 nm, Em. 590
131 nm) using a BMG Polarstar plate reader.

132 **Rapid purification of mAGP**

133 Rapid purification of soluble peptidoglycan from small cultures was carried out using a
134 modified phenol extraction protocol[18]. Mycobacterial cells were grown to mid-exponential
135 phase and collected by centrifugation. These were washed with cold phosphate-buffered saline
136 (PBS) and resuspended in PBS and the cells were lysed in a Percellys Evolution Bead Beater
137 at 5,000 rpm for 3 min. The lysate was then transferred to glass culture tubes to which 2 mL of

138 98% phenol was added and vortexed for 1 min. This was heated for 1h at 70 °C, allowed to cool
139 and the insoluble material was collected by centrifugation at 5,000 rpm. The aqueous phase was
140 removed and 4 mL of methanol was added. This was vortexed and centrifuged again. Finally,
141 the pellet was washed 3 times with methanol and once with water before being frozen or used
142 for subsequent enzymatic treatment.

143 **Large-scale purification of PG**

144 Purification of PG from *M. smegmatis* was achieved following established protocols[19].
145 Six-liters of *M. smegmatis* were grown to mid-exponential phase at which point they were
146 harvested by centrifugation, resuspended in a minimal volume of PBS and lysed by sonication.
147 The resulting lysate was brought to 4% SDS and boiled under reflux for 3 h. The insoluble
148 material was collected by centrifugation and washed with water until the SDS was completely
149 removed (at least 7 washes) to yield mycolyl-arabinogalactan-peptidoglycan (mAGP). The
150 mAGP was incubated for 4 days in 0.5% KOH in methanol at 37 °C before being washed three
151 times with methanol. The mycolic acids were extracted with 3 washes of diethyl ether. The
152 phosphodiester linking the AG-PG complex was cleaved using 0.2 M H_2SO_4 and the PG was
153 separated from the solubilized AG by centrifugation prior to neutralization with $NaCO_3$ and
154 washed with water 3 times. The insoluble PG pellet was sequentially digested with α-amylase
155 (100 μg•mL^{-1}), DNase (10 μg•mL^{-1}) and RNase (5 μg•mL^{-1}) for 8h before proteinase K (100
156 μg•mL^{-1}) digestion overnight at 37 °C. The PG pellet was resuspended in a minimal volume of
157 1% SDS and boiled under reflux for 3 h before the SDS was removed by centrifugation and
158 washing with water (at least 7 times). The resulting material was lyophilized and stored at -20
159 °C until it was needed.

160 Digestion of cell wall material with mutanolysin was carried out overnight at 37 °C in
161 20 mM ammonium acetate buffer (pH 6.0) with continuous mixing. Following digestion
162 solubilised muropeptides were isolated using graphitized carbon solid-phase-extraction

163 cartridges as previously described[20]. Purified fractions were evaporated to dryness and the
164 concentration of reducing sugars in the pool of soluble muropeptides was assessed using the 3-
165 methyl-2-benzothiazolinone hydrazone (MBTH) assay[21].

166 **Synthesis of 4MU-D-lactate**

167 Instead of the 2- or 3- step protocols published for the synthesis of 4MU-D-lactate previously,
168 we used a simplified one step method[22,23]. 1.5 g of (s)-(-)-bromopropionic acid was added to 1
169 g of 4-methylumbelliferone stirring in 40 mL anhydrous dimethylformamide and 0.75 g
170 Cs_2CO_3. This was stirred at room temperature over-night and the product was extracted three
171 times with water/ethyl-acetate and the organic phase was dried over sodium sufate. The organic
172 phase was then filtered and evaporated to dryness. The product was subsequently purified using
173 silica chromatography and was dried as a crystalline white solid.

174 **Turn-over of 4MU reporter compounds by *M. bovis* BCG**

175 To test turn-over of 4MU-Glc*N*Ac or 4MU-D-lactate by whole cells, 100 µL of a mid-
176 exponential culture (OD_{600} = 0.6) was added to a sterile 96 well plate in Sauton's minimal media
177 supplemented with 0.05% Tween and 1% glycerol in addition to 1 mM 4MU-D-lactate or 4MU-
178 Glc*N*Ac. Similar controls lacking cells or the reporter compound were included as well. This
179 was incubated at 37 °C and mixed at 300 r.p.m. Each day the fluorescence of the sample was
180 read on a BMC PolarStar microplate reader (Ex. 355 nm; Em 460 nm) with a constant gain
181 setting.

182 **Turn-over of *M. bovis* BCG PG *in vitro***

183 Cultures of *M. bovis* BCG wild-type, Δ*lpqI*, and Δ*lpqI*::*lpqI* were grown to an OD_{600} of
184 0.6 in the presence of 10 µCi ^{3}H *meso*-diaminopimelic acid (DAP), at which point they were
185 collected by centrifugation, washed 3 times with sterile media and diluted to 0.01 in fresh
186 culture flasks. Periodically a sample of 0.5 mL was taken, and the cells were collected by

187 centrifugation. The spent medium was mixed with 10 mL scinitilation fluid and counted using
188 a liquid scintillation counter. The cell pellet was re-suspended in 10% SDS, boiled for 20 min,
189 and centrifuged again. The cell-wall material was then resuspended in 1 mL scintillation fluid
190 and the material was counted in a liquid scintillation counter. The counts of the cell wall and
191 the media were added together to give total ^{3}H DAP in each culture and the data is presented as
192 a percentage of that total. During the course of the experiment the OD_{600} of the culture was
193 monitored daily. All measurements are from three biological replicates.

194 **Cloning and purification of Rv0237**

195 Rv0237 was cloned from *M. tuberculosis* H37Rv into the T-A site of the Champion
196 pET-SUMO expression plasmid (Invitrogen) using standard PCR conditions with the
197 Rv0237SUMOF and Rv0237SUMOR primers. For production of Rv0237 1 L of *E. coli* BL21
198 [pRv0237] grown in Terrific Broth to an OD_{600} of 0.6, chilled to 20 °C and induced with 1 mM
199 IPTG and grown for a further 18 h before being collected by centrifugation**.** Cells were
200 resuspended in 25 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole pH 7.8 and lysed *via* three
201 passages through a French pressure cell. The protein was purified using standard IMAC
202 procedures with washes of lysis buffer, lysis buffer including 50 mM imidazole and finally
203 eluted with 500 mM imidazole in lysis buffer. Eluted protein was dialysed exhaustively against
204 25 mM Bis-Tris, 100 mM NaCl pH 7.8 in the presence of recombinant Ulp1 protease which
205 specifically cleaves the His_6-SUMO tag. Digested protein was subjected to a second IMAC
206 column (1 mL HisTrap FF, GE Healthcare) and the flow-through fraction was found to contain
207 pure, un-tagged Rv0237. Purified protein was dialysed into 25 mM Bis-Tris pH 6.5, 100 mM
208 NaCl.

209 **Crystallography**

210 Prior to crystallization, LpqI was concentrated to 20 mg•mL^{-1} in 25 mM Bis-Tris pH
211 7.5. LpqI crystals were grown by the sitting-drop vapour diffusion method by mixing an equal

212 volume of protein solution with 1.1 M sodium malonate, 0.1M HEPES, 0.5% v/v Jeffamine
213 ED-2001 (pH 7.0). Crystals were cryo-protected with a saturated solution of sodium malonate
214 and plunge frozen in liquid nitrogen. X-ray data was collected at the Diamond Light Source,
215 Oxford. Data were processed using XiaII and file manipulations were performed using the
216 CCP4 suite of programs. The structure was phased by molecular replacement using the
217 previously released, but unpublished *M. smegmatis* LpqI structure (PDB: 4YYF) using the
218 program PHASER. The structure was subsequently auto-built in PHENIX and the remaining
219 parts were built in COOT with further refinement using PHENIX and PDB-REDO.

220 **Kinetic characterisation of Rv0237**

221 Purified Rv0237 was evaluated for glycoside hydrolase activity using a variety of
222 substrates. As an initial screening assay, Rv0237 was incubated at 1 μM with either 4-
223 methylumbeliferyl or *p*-nitrophenyl derivatives of a variety of sugars as listed in Figure 4a in
224 Bis-Tris pH 7.5, 100 mM NaCl at 37 °C. The release of *p*-nitrophenol was followed by change
225 in absorbance at 420 nm while production of 4-menthylumbelliferone was monitored by
226 fluorescence as above in a BMG Polarstar spectrophotometer. Kinetic characterisation of
227 Rv0237 was conducted using varying concentrations of 4MU-Glc*N*Ac. The raw data were
228 compared to standards of 4-methylumbelliferone. All data were analysed using GraphPad Prism
229 7.

230 To evaluate the ability of the enzyme to degrade fragments derived from PG, *M.*
231 *smegmatis* PG was digested with mutanolysin and soluble fragments were prepared and
232 quantified as above. Reactions including 1 μM Rv0237, 0.5 mM PG fragments in 25 mM
233 ammonium acetate buffer pH 6.5 were incubated for 18h at 37 °C. In parallel reactions were
234 carried out using *p*NP-Glc*N*Ac in order to monitor enzyme activity visually. The reactions were
235 then evaluated by TLC (Silica 60 F_{254}, Merck, Germany) using a mobile phase consisting of 1-

236 butanol, methanol, ammonium hydroxide and water at a ratio of 5:6:4:1. TLCs were stained
237 with α-naphthol and developed by charring.

238 **Infection of bone marrow-derived macrophages**

239 BMDM were differentiated from bone marrow cells obtained from femurs and tibiae of
240 C57BL/6 mice cultured in the presence of L-cell conditioned medium, as described before[24].
241 One million cells were infected with each *M. bovis* BCG strain at a multiplicity of infection
242 (moi) of 2 bacteria: 1 macrophage. Four hours after infection, cells were washed 4 times with
243 PBS (GIBCO) to remove extracellular bacteria. The adherent cells were cultured at 37°C in 1
244 mL of cDMEM in the presence or absence of 100 U/mL of IFN-γ for 96 hours. Four or 96 hours
245 post-infection, 0.1% saponin (Sigma-Aldrich) in PBS was added to the wells and the cells were
246 incubated at RT for 10 minutes to release intracellular bacteria. The number of viable bacteria
247 was determined by plating 10-fold serial dilutions of the saponin treated cell suspensions in
248 Middlebrook 7H11 supplemented with 10% OADC and 0.5% glycerol. Viable bacteria were
249 determined by CFU enumeration after 21-28 days of incubation at 37°C[24].

250 # Results

251 **Peptidoglycan Recycling Genes in *Mycobacteria***

252 The genome of *M tuberculosis* encodes many lytic enzymes, including at least five
253 Resuscitation Promoting Factors (Rpfs) and greater than 10 peptidases and amidases in addition
254 to penicillin binding proteins with potential lytic activities[25]. The Rpfs are most likely lytic
255 transglycosylases with the product of RpfB having been recently confirmed as a GlcNAc-1,6-
256 anhydroMurNAc disaccharide[26]. While *M. tuberculosis* does appear to encode at least one
257 GH25-lysozyme, Rv2525, its activity has not been conclusively demonstrated[27]. A recent
258 comparative study of PG-active enzymes in mycobacteria indicated that while significant
259 differences exist, enzymes that can likely degrade all of the major covalent linkages of PG are

260 encoded in the genomes of all mycobacteria[25]. The products of most of these enzymes have not
261 been experimentally demonstrated, however their conservation underscores the importance of
262 PG-remodeling during growth and division of mycobacteria.

263 Most autolytic enzymes produce small PG-metabolites (muropeptides), indicating that
264 mycobacteria should generate these molecules during the course of normal growth. Indeed,
265 soluble PG fragment release has been observed for both *M. smegmatis* and *M. tuberculosis in*
266 *vitro*[18,28]. We hypothesized that a recycling system for these muropeptides is likely to also exist
267 in mycobacteria and analyzed the genomes of several mycobacteria for known PG-recycling
268 systems including the recently discovered systems of *Pseudomonas putida* and *Tannerella*
269 *forsythia* (Table S1, Figure 1)[10,29]. BLAST analysis of the *Corynebacterium glutamicum, M.*
270 *tuberculosis, Mycobacterium leprae* and *Mycobacterium bovis* BCG genomes indicates that
271 they lack genes related to any known muropeptide import proteins, PG-metabolite
272 phosphorylation systems, and *murQ*. The only sugar-kinase orthologs identified in the genome
273 have previously been characterized as glucose-kinases although they have not been directly
274 tested for amino sugar-phosphotransferase activity[30]. This contrasts sharply with *M. smegmatis*
275 for which an apparently complete "classical" muropeptide recovery system exists, making it a
276 poor PG metabolism model for *M. tuberculosis* (Table S1). The *M. tuberculosis, M. leprae, C.*
277 *glutamicum and M. bovis* BCG genomes do, however, appear to encode orthologs of NagA and
278 NagZ. These enzymes are predicted to be an *N*-acetylglucosamine-6-phosphate N-deacetylase
279 and a GH3-family β-*N*-acetylglucosaminidase respectively[31,32]. NagZ in particular is typically
280 associated with PG recycling, whilst NagA is typically associated with the assimilation of
281 Glc*N*Ac regardless of the source.

282 **Utilisation of peptidoglycan components by mycobacteria**

283 Prior research has shown that most mycobacteria are unable to use Glc*N*Ac as a sole
284 carbon source, with *M. smegmatis* being one of the notable exceptions[33]. Furthermore, amino

285 acids including L-Ala, L-Glu, and L-Asp have previously been shown to serve as nitrogen
286 sources for *M. tuberculosis* H37Rv[34]. To our knowledge, recycling of Glc*N*Ac or Mur*N*Ac has
287 not been reported, nor has recycling been evaluated for soluble PG fragments. To evaluate this,
288 *M. bovis* BCG was cultured in minimal media supplemented with glycerol (1% v/v) or Mur*N*Ac
289 (0.2% w/v) in Sauton's minimal media with constant aeration (Figure 2a). To confirm that this
290 was not a phenotype specific to *M. bovis* BCG we also evaluated the ability of *M. tuberculosis*
291 H37Rv to grow on the same carbon sources with identical results (Figure 2b) Intriguingly this
292 growth was heavily dependent on the availability of oxygen. In contrast, growth on glycerol
293 was unaffected by this change (Figure 2c). To further evaluate the potential for simple re-use
294 rather than metabolism of Glc*N*Ac we tested the ability of *M. bovis* BCG to incorporate ^{14}C
295 Glc*N*Ac into whole cells. Under different growth conditions (rich medium, carbon-poor
296 medium, aerated cultures, static cultures) we were unable to detect significant amounts of
297 Glc*N*Ac being taken up by *M. bovis* BCG. In all cases the c.p.m. of the label in whole-cells was
298 less than that of unlabeled controls.

299 **Mechanism of Mur*N*Ac metabolism**

300 The ability of *M. tuberculosis* and *M. bovis* BCG to grow on Mur*N*Ac was surprising
301 and so we evaluated the biochemical processing steps associated with Mur*N*Ac utilization.
302 Mur*N*Ac is a combination of Glc*N*Ac and D-lactate joined by an ether linkage. This suggests
303 that the bacterium is likely either using the Glc*N*Ac moiety for glycolysis, or shunting the lactate
304 derived from Mur*N*Ac into the TCA cycle. We tested this inhibiting by glycolysis with 2-
305 deoxyglucose (2DG) in cultures grown using Mur*N*Ac, glucose and glycerol as sole carbon
306 sources (Figure S1). These data suggested that the pathway of Mur*N*Ac utilization did not
307 require glycolysis and indicated that lactate instead was likely serving as a carbon source.
308 Consistent with this, when used as a sole carbon source, growth on L-lactate and Mur*N*Ac was
309 O_2 dependent while D-lactate was better utilized under static, 5% CO_2 culture conditions, where
310 Mur*N*Ac could not be used as a carbon source (Figure 2c).

311 These data allow us to hypothesize a mechanism by which *M. bovis* BCG metabolises
312 Mur*N*Ac. Given that metabolism of L-lactate and Mur*N*Ac are O_2-dependent, we anticipate that
313 use of Mur*N*Ac follows cleavage of the D-lactate from Mur*N*Ac *via* an inverting mechanism to
314 produce L-lactate and Glc*N*Ac. In this case, the O_2-dependency on Mur*N*Ac metabolism is
315 likely the result of an O_2-depenedent lactate monooxygenase. Consistent with this, two O_2-
316 dependent L-lactate monooxygenases have been identified in *M. tuberculosis* (Rv0694,
317 Rv1872c)[35]. Generation of free lactate by the bacterium would require the activity of a lactyl-
318 etherase enzyme. To test for the presence of this activity we synthesized a 4MU-D-lactate
319 derivative to serve as a reporter-analog of Mur*N*Ac (Figure S2). Consistent with the presence
320 of a lactyl etherase, cultures of *M. bovis* BCG were able to release 4MU from this compound
321 during the course of growth (Figure 2d).

322 **Uptake of PG metabolites by mycobacteria**

323 While our data strongly support metabolism of Mur*N*Ac by *M. bovis* BCG, confirmation
324 of PG-recycling requires demonstration of the uptake of muropeptides by the bacterium. To
325 investigate this, we generated radio-labelled muropeptides and tested them in whole-cell uptake
326 assays to determine if mycobacteria are competent for recycling this more complex substrate.
327 Muropeptides had to be generated in *M. smegmatis* due to the inability of *M. bovis* BCG to take-
328 up ^{14}C Glc*N*Ac under the conditions we tested. As shown in Figure 3a, *M. bovis* BCG was able
329 to incorporate approximately 4% of the muropeptide-associated ^{14}C radio-label added to the
330 culture. We next sought to determine if components of the stem-peptide were also recycled.
331 The above experiments were repeated using ^{3}H-DAP-labelled muropeptides. This material was
332 also incorporated into whole cells at a rate of approximately 7% of the added label (Figure 3a).
333 We next evaluated the turn-over of muropeptides in whole cells using ^{3}H-DAP due to the
334 inability of *M. bovis* BCG to incorporate ^{14}C Glc*N*Ac into its cell wall. As shown in Figure 3c,
335 *M. bovis* BCG very slowly releases DAP to the culture media *in vitro*. Consistent with a PG-
336 recycling system we also found that soluble PG could serve as a sole carbon source for *M. bovis*

337 BCG under aerated conditions (Figure 3d). Together, these results indicate that pathogenic
338 mycobacteria possess the biochemical capacity to recycle components of their cell wall.

339 **Biochemical and structural characterisation of LpqI**

340 In previously characterized PG-recycling systems free amino sugars are by a glycoside
341 hydrolase family 3 enzyme named NagZ[36]. The CAZy glycoside hydrolase family 3 (GH3) is
342 a large group of enzymes which have hydrolytic and phosphorylytic activity to remove β-linked
343 sugars from proteins and polysaccharides[37,38]. The β-*N*-acetylglucosaminidase sub-family
344 including all known NagZ enzymes utilize a conserved Asp-His catalytic dyad which has been
345 well characterized[39,40]. A BLAST search of the *M. tuberculosis* H37Rv genome revealed only
346 one NagZ ortholog, which was previously named LpqI in light of its identification as a
347 lipoprotein including an appropriately positioned lipobox at the N-terminus of the protein[41]. As
348 a lipoprotein LpqI is expected to be found attached to the periplasmic face of the cytoplasmic
349 membrane, which is consistent with proteomics results[41]. LpqI has also been identified as a
350 likely mannosylated glycoprotein in a proteomics screen using ConA chromatography[42]. The
351 *lpqI* gene is found in all mycobacteria with sequenced genomes including *M. leprae* which has
352 a substantially reduced genome (Figure 1, Table S1, Figure S5).

353 We cloned, expressed and purified the LpqI protein from *M. tuberculosis* H37Rv using
354 an N-terminal His_6-SUMO tag which was subsequently cleaved from the protein. While LpqI
355 bears significant sequence similarity to known β-*N*-acetylglucosaminidases, recent studies have
356 demonstrated that divergent activities for this sub-family of enzymes are possible[38]. These
357 activities included the ability to release sugars other than Glc*N*Ac from reporter substrates and
358 apparent phosphoroyltic activity. We first sought to determine if the protein was in fact a β-*N*-
359 acetylglucosaminidase by testing its activity on a variety of substrates including many sugars
360 that would be found in the cell wall of mycobacteria. Using convenient reporter sugars we
361 assessed the ability for the enzyme to release *p*-nitrophenolate or 4-methylumbeliferone from

362 conjugated arabinose, galactose, galactosamine, arabinofuranose, glucose, mannose,
363 mannosamine, glucosamine and *N*-acetylglucosamine (Figure 4a). While not exhaustive, this
364 set of sugars covers most major modifications to the cell wall including the AG itself, *O*-
365 mannose modifications of proteins, Gal*N*Ac modification of arabinan, the rhamnose-linker
366 sugar of AG and the Glc*N*Ac and Glc*N* found in PG. The only detectable activity for LpqI was
367 with Glc*N*Ac-containing substrates. Critically, this sugar is only found in the backbone of PG
368 and a small amount in the linker unit (Mur*N*Ac-6-P-Rha-Glc*N*Ac-galactan) between PG and
369 arabinogalactan. We then evaluated the Michaelis-Menten kinetics of LpqI using 4MU-Glc*N*Ac
370 as a substrate with a similar k_{cat} ($2.8 \times 10^{-2} \pm 0.04 \times 10^{-2}$ $\bullet s^{-1}$) and K_m (106 ± 5 μM) as observed
371 for other NagZ enzymes using this substrate (Figure 4b) [39]. In a similar assay we were also able
372 to show that LpqI releases Glc*N*Ac from soluble PG fragments (Figure 4c). While hydrolytic
373 activity has been reported for most NagZ-type enzymes, a recent report suggested that β-*N*-
374 acetylglucosaminidases from the GH3 family are in fact phosphorylases[38]. Another GH3 β-*N*-
375 acetylglucosaminidase was recently reported to lack this activity, suggesting that it may not be
376 a general property of the family[43]. We tested the activity of the enzyme under the same
377 conditions as reported previously for Nag3 from *Celulomonas fimi* and found that there was no
378 detectable difference with our observed hydrolytic activity. The product of the reaction also co-
379 migrated with Glc*N*Ac on TLCs and not Glc*N*Ac-1-P (Figure S3).

380 To further validate its role in PG-recycling we solved the 1.96 Å crystal structure of
381 LpqI (PDB code: 6GFV; Figure 4d, S4, Table S3). LpqI consists of a single TIM-barrel domain
382 similar to cytoplasmic Gram-negative orthologs but lacks the C-terminal domain associated
383 with extracellular NagZ enzymes from some Gram-positive bacteria (Figure S4). Alignment of
384 LpqI with the NagZ/Glc*N*Ac/1,6-anhydroMur*N*Ac complex from *Pseudomonas aeruginosa*
385 ($NagZ_{Pa}$; PDB:5G3R) or NagZ from *Bacillus subtilis* (PDB:4GYJ) resulted in a root-mean-
386 square deviation of 0.96 Å and 1.01 Å respectively (Figure S4). Superposition of the post-

387 cleavage $NagZ_{Pa}$ complex with LpqI indicates that the appropriate coordinating residues for
388 substrate recognition are intact in LpqI, supporting its role in PG-recycling (Figure 4d).

389 **Characterisation of a Δ*lpqI* mutant**

390 To evaluate the role of LpqI in muropeptide recovery, we constructed a mutant strain of
391 *M. bovis* BCG lacking *lpqI* using specialized transduction and confirmed the mutant by PCR[16].
392 A whole cell β-*N*-acetylglucosaminidase assay clearly shows that *M. bovis* BCG Δ*lpqi* is devoid
393 of β-*N*-acetylglucosaminidase activity as the amount of 4MU released is not significantly
394 different from the spontaneous release in sterile media (Figure 3b). This deficiency is
395 complemented by replacement of the *lpqI* gene at a distal chromosomal location under the
396 control of its native promoter (Δ*lpqi*::*lpqI*) and is not complemented by the empty vector
397 (Δ*lpqi*::EV) (Figure 3b). Growth of Δ*lpqi in vitro* is unaltered as compared to the wild-type
398 (Figure 3c).

399 The order in which muropeptides are recycled, and the chemical structure of the
400 recycled material is critical for the immune sensing of the bacterium. To determine the order of
401 PG-recycling steps, we first determined the impact of the loss of *lpqI* on the recycling of cell
402 wall material. We repeated the radio-label incorporation assay described above with the mutant
403 and observed that the Δ*lpqI* was able to incorporate ^{3}H stem-peptides from soluble PG as
404 efficiently as the wild-type (Figure 3b). Consistent with these observations, when we followed
405 release of pre-labelled cells for release of ^{3}H DAP into the culture media, we observed no
406 significant differences between the wild-type and the Δ*lpqI* strain (Figure 3c). This experiment
407 reported on the recycling of stem-peptides, however it did not indicate if the mutant strain was
408 still recycling Mur*N*Ac. To test this directly we evaluated the ability of the Δ*lpqI* strain to grow
409 on Mur*N*Ac, glycerol and PG. The Δ*lpqI* strain was not deficient for growth on Mur*N*Ac or
410 glycerol, however unlike the wild-type strain it was unable to grow on PG as a sole-carbon
411 source (Figure 3d). Similarly, the Δ*lpqI* strain incorporated significantly fewer ^{14}C-Glc*N*Ac-

412 labelled muropeptides (Figure 3a). Together these data indicate that *in vitro lpqI* is required for
413 amino-sugar recycling, but is not necessary for stem-peptide recycling or release.

414 Given that NagZ-like proteins have been found to play a role in β-lactam sensitivity in
415 other bacteria we sought to determine the antibiotic sensitivity of the Δ*lpqI* strain. In contrast
416 to inhibition of *P. aeruginosa* NagZ, deletion of *lpqI* resulted in a significant increase in survival
417 for lysozyme and all cell-wall active antibiotics tested (Figure 5a-d)[44]. A smaller impact on
418 survival in the presence of the protein synthesis inhibitor chloramphenicol was observed
419 (Figure 5e). This increase in resistance is not likely due to a change in cell-wall permeability as
420 determined by ethidium bromide uptake (Figure 5f).

421 ***In vitro* characterization of a Δ*lpqI* mutant**

422 We next sought to determine the impact of the loss of *lpqI* on host responses to infection.
423 Bone-marrow derived macrophages were infected with *M. bovis* BCG WT, Δ*lpqi*, Δ*lpqi*::*lpqi*
424 and Δ*lpqi*::EV at an MOI of 2 and evaluated for total colony forming units (CFU) at 4h and 4
425 days post-infection. While no significant differences were observed at 4 h post-infection, a log-
426 increase in CFUs was observed in the Δ*lpqI* and Δ*lpqI*::empty vector strains as compared to the
427 wild-type or complemented mutant (Figure 6). This striking result indicated that although
428 bacteria uptake by macrophages is independent of LpqI, growth of *M. bovis* BCG in the
429 macrophage is controlled in a *lpqI*-dependent manner.

430 As a result of the apparent increase in fitness of a Δ*lpqI* strain under the conditions tested
431 we analyzed all available *M. tuberculosis* genomic sequences using BLAST for mutations in
432 the *lpqI* coding or promoter regions. We were unable to identify any deletions/mutations in the
433 promoter region for this protein and all identified mutations in the coding sequence were in
434 positions unlikely to be involved in catalysis or binding or were conservative mutations and
435 thus unlikely to impact function.

436 # Discussion

437 In an attempt to develop diagnostic media for the identification of mycobacteria, several
438 groups in the 1960s observed that *M. tuberculosis* and most other mycobacteria could not
439 metabolise Glc*N*Ac as a sole carbon-source[33,34]. This, along with the absence of known PG
440 recycling-associated genes lead to the assumption that PG recycling is absent in pathogenic
441 mycobacteria. Our sole-carbon source assays indicate that while the bacteria are unable to
442 metabolise Glc*N*Ac, surprisingly they can metabolise Mur*N*Ac (Figure 2). This is despite the
443 fact that they lack an ortholog of the only known lactyl-etherase, MurQ which cleaves an
444 otherwise stable lactyl-ether in the cytoplasm of most model organisms (Figure 1). Our data
445 indicate that rather than using the Glc*N*Ac portion of the sugar, the bacteria are cleaving the
446 lactyl-ether and capable of metabolising the liberated lactate. During our study we found that
447 *M. bovis* BCG was only able to grow on Mur*N*Ac under aerated conditions. This was also found
448 to be the case for L- but not D-lactate which served as a much better carbon source under O_2
449 limiting conditions. As Mur*N*Ac is a combination of D-lactate and Glc*N*Ac, we can predict that
450 the lactyl etherase acting on Mur*N*Ac is likely proceeding *via* an inverting mechanism. The
451 presence of a specific lactyl-etherase is supported by the turnover of a 4MU-D-lactate reporter
452 compound by *M. bovis* BCG. The O_2 dependence of this growth is intriguing as N-glycolylation
453 is also an O_2-dependent activity, suggesting significant alterations to PG metabolism in hypoxic
454 vs. aerobically growing mycobacteria[45]. Consistent with this observation, Rv0237 has a 2-fold
455 upregulation during re-aeration after re-activation from non-replicating persistence in the
456 Wayne hypoxia model[46].

457 Autolytic enzymes that cleave the glycan backbone of PG such as glucosaminidases,
458 lytic transglycosylases and lysozymes generally produce disaccharides. As such, free Mur*N*Ac
459 is unlikely to be generated by the known complement of autolytic enzymes in TB. We therefore
460 sought to identify the biochemical source of free amino-sugars which would feed a PG-

461 recycling system. To do this, we biochemically and structurally characterised the predicted
462 mycobacterial NagZ ortholog, LpqI demonstrating that it is an authentic β-*N*-
463 acetylglucosaminidase which is active against PG fragments. Consistent with a role in PG-
464 recycling, *M. bovis* BCG Δ*lpqI* is unable to grow on soluble PG as a sole carbon source, while
465 recycling of the stem-peptide is unaltered in this mutant (Figure 3). Furthermore, uptake of
466 radio-labelled stem peptides was unchanged in the Δ*lpqI* mutant whereas ^{14}C Glc*N*Ac-
467 muropeptides show a significant decrease in incorporation (Figure 3). Together, these data
468 demonstrate that *M. bovis* BCG and *M. tuberculosis* remove the stem-peptide from PG-
469 fragments prior to disaccharide cleavage and lactyl-ether removal (Figure 7). The processing
470 of Glc*N*Ac-Mur*N*Ac by LpqI prior to lactyl-ether cleavage is also supported by our LpqI crystal
471 structure in which the lactate-binding residue R67 from the *P. aeruginosa* structure is conserved
472 (LpqI: R130).

473 The fate of Glc*N*Ac in this pathway remains unclear, although our data and prior
474 observations suggest that the bacteria do not re-use this sugar. This is surprising given the
475 conservation of the *nagA* (Rv3332) gene in mycobacteria, however it is possible that an
476 alternative pathway exists which involves intermediates not generated under the conditions we
477 have tested. This is hinted at with our ^{14}C-labelled muropeptides where incorporation of the
478 labelled-Glc*N*Ac is not expected given the lack of Glc*N*Ac utilisation by the cells. It is likely
479 that at least some portion of the labelled material is labelled at Mur*N*Ac rather than Glc*N*Ac
480 and that the sugar moiety is in fact used as some alternative reaction product upon cleavage of
481 the lactyl-ether. Bacterial etherases comprise a diverse number of mechanisms and potential
482 reaction products and so a product other than free Glc*N*Ac is entirely possible[47]. We are
483 currently trying to identify and characterise this enzyme.

484 The recycling of bacterial PG has immense implications for the host-pathogen
485 relationship. PG has been shown to be a pathogen-associated molecular pattern and is detected
486 by many different specialised host receptors[8,48]. Of most relevance to *M. tuberculosis* is the

487 NOD2 receptor which senses intracellular muramyl-dipeptide (Mur*N*Ac-L-Ala-D-*iso*Glu) as a
488 minimal motif[8]. The immunogenicity of Freund's complete adjuvant, for example, is driven by
489 the presence of mycobacterial PG and its *N*-glycolyl modification[49]. Despite this, Hansen and
490 colleagues observed that the detection of *M. tuberculosis* by the immune system *via* Nod2 is
491 weaker than expected, with equal preparations of dead bacteria having substantially more
492 NOD2-stimulatory activity than wild-type bacteria[50]. The authors of that study speculated that
493 this was either due to active repression of the immune system or a reduction in the amount of
494 free NOD2-stimulatory effectors in live bacteria.

495 In our work, we have shown that mycobacteria recycle their PG by first cleaving the
496 stem peptide from the glycan backbone, and subsequently recycle the MurNAc portion of the
497 glycan, removing the D-lactate. This step-wise activity, starting with stem peptide removal,
498 would dramatically reduce the release of NOD2-stimulatory molecules, especially given that
499 this activity is happening beneath the mycomembrane, where diffusion of muropeptides is
500 expected to be highly restricted. Heat-killing of these bacteria would allow host-derived
501 lysozymes to release muropeptides and for those muropeptides to be able to diffuse and
502 stimulate NOD2 and other receptors. In line with this, our preliminary analysis suggest that
503 absence of LpqI does not alter the production of cytokines by infected bone-marrow-derived
504 macrophages (data not shown).

505 Deletion of the *lpqI* gene from *M. bovis* BCG yielded several surprising observations.
506 Impaired PG recycling has resulted in a strain that is more resistant to both lysozyme and several
507 antibiotics while not affecting growth *in vitro.* We are currently investigating the mechanistic
508 basis for this, though it is not likely due to a change in permeability of the cell wall (Figure 5).
509 In other bacteria cell wall damage can trigger various stress responses, and so it is likely that a
510 build-up of Glc*N*Ac-Mur*N*Ac disaccharides may trigger a stress-like response in
511 mycobacteria[51]. Consistent with this *lpqI* is encoded adjacent to a universal stress response
512 protein in several mycobacteria (Figure S5).

513 Loss of this gene has also resulted in a substantial increase in growth in bone-marrow
514 derived macrophages (Figure 6) suggesting that cell-wall turnover may act as a growth-rate
515 modulator *in vivo.* Despite these apparent fitness advantages, the *lpqI* gene appears to be intact
516 in virtually all mycobacteria for which sequence data is publicly available, and observed
517 mutations are unlikely to impact catalysis (Figure S5). This suggests that there is a fitness cost
518 to the inactivation of this gene and warrants further investigation, perhaps in whole organismal
519 models. One possibility is that under stress-conditions mycobacteria may be able to scavenge
520 PG fragments from nearby dead cells allowing a small population to re-grow following mass
521 lysis. This is consistent with the observation that PG can lead to resuscitation of dormant
522 mycobacteria[52]. Alternatively, Δ*lpqI*-driven excessive growth in the macrophage may prevent
523 the development of a stable, long-term infection. PG recycling has also been shown to be critical
524 for Gram-positive bacteria in stationary phase, though our data do not support this requirement
525 for *M. bovis* BCG, it is possible that it is more important in the host[53].

526 In conclusion, we have identified for the first time a PG recovery pathway in pathogenic
527 mycobacteria. We have shown that this occurs in a step-wise fashion by removing stem-peptide
528 from PG and subsequently cleaving the PG-disaccharide and finally releasing the D-lactate from
529 free Mur*N*Ac, most likely *via* an inverting mechanism. Finally, recycling of PG by these
530 bacteria is important for lysozyme and antibiotic tolerance, while deletion of this system results
531 in a significant growth advantage for these bacteria in macrophages.

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Author Contributions

Conceived of the study: P.J.M. Conceived and designed the experiments: P.J.M., G.V.M., M.S., A.L.L., G.S.B. Performed the experiments: P.J.M., A.R.M., I.T.C, N.V., M.J., M.C. Analysed the data: P.J.M., A.R.M., I.T.C, N.V., M.C. G.V.M., M.S., A.L.L., G.S.B. Wrote and edited the paper: P.J.M, G.V.M., M.S., A.L.L., G.S.B.

Figures and Tables

a)

		AmpG or P-transport	Amino sugar Recycling		Peptide Recycling	
			MurNAc	GlcNAc	Amidase	Import
Gram -	*E.c.*	+	+	+	+	+
	P.p.	+	+	-	+	+
Gram +	*S.a.*	+	+	+	+	+
	B.s.	+	+	+	+	+
Corynebacteria	*C.g.*	-	-	-	+	+
	M.s.	+	+	+	+	+
	M.t.	-	-	-	+	+
	M.l.	-	-	-	+	+
	M.b.	-	-	-	+	+

b)

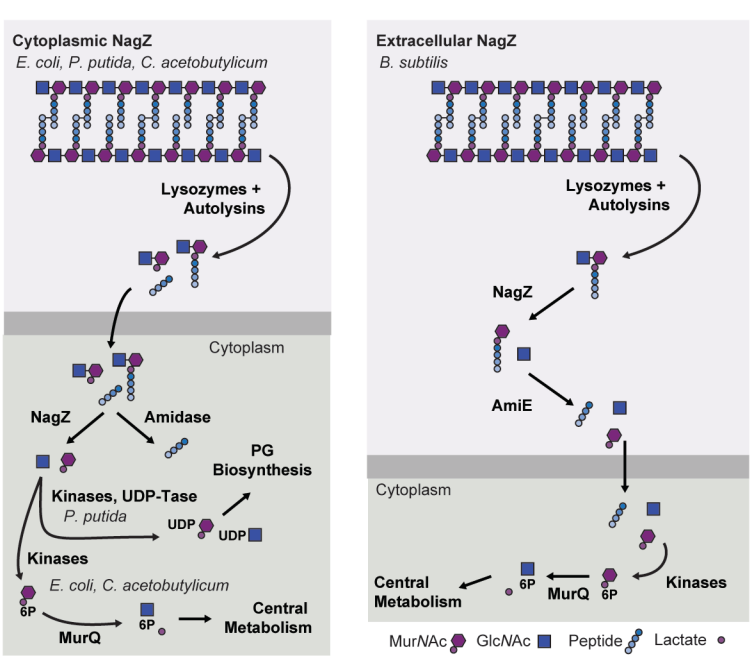

Figure 1. Overview of PG-recycling. a) The genes involved in PG-recycling fall into functional groups that are typically conserved amongst closely related species, with two major Mur*N*Ac recovery systems so far identified (AnmK/MurQ and AngK/MurU). **b)** For a complete listing of gene conservation see Table S1. The PG recycling machinery is variable with respect to the localisation of NagZ and the subsequent conversion to Glc*N*Ac-1P or UDP-Glc*N*Ac/Mur*N*Ac. All known Mur*N*Ac recovery systems that sustain bacterial growth (as opposed to strictly recycling e.g. *P. putida*) terminate at MurQ in the cytoplasm. *E.c.* – *E. coli, P.p* – *P. putida, S.a.* – *Staphylococcus aureus, B.s.* – *Bacillus subtilis, C.g.* – *Corynebacterium glutamicum, M.s.* – *M. smegmatis, M.t.* – *M. tuberculosis, M.l. M. leprae, M. b.* – *M. bovis* BCG.

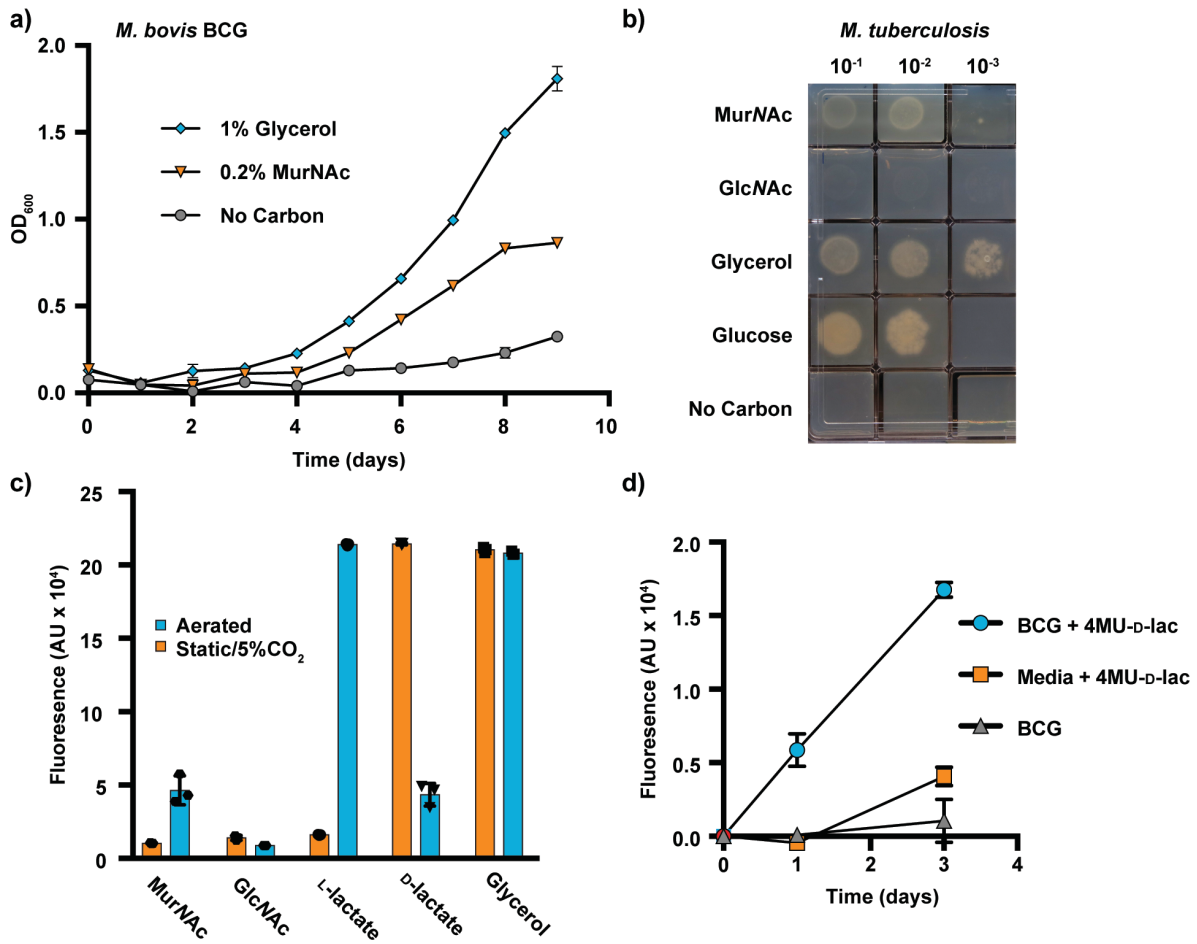

Figure 2. *M. tuberculosis* and *M. bovis* BCG are able to recycle Mur*N*Ac. a) *M. bovis* BCG WT was inoculated at a starting OD_{600} of 0.1 in minimal media containing glycerol and tyloxopol, Mur*N*Ac or no carbon and growth was monitored daily by taking OD_{600} readings at the indicated concentrations (n = 3). **b)** *M. tuberculosis* H37Rv was washed and then serially diluted into fresh carbon-free minimal media. 10 µL of each dilution was spotted onto Sauton's agar containing the indicated carbon sources at 5 mM. **c)** Growth of *M. bovis* BCG on 5 mM Mur*N*Ac, Glc*N*Ac, L-lactate, D-lactate, and glycerol was evaluated in aerated or static 5% CO_2 cultures using a resazurin assay (n = 3). **d)** Mid-exponential *M. bovis* BCG was grown in minimal media with 5 mM glycerol including 1 mM 4MU-D-lactate with constant agitation. At the indicated times the 4MU fluorescence of the samples was determined (n = 3).

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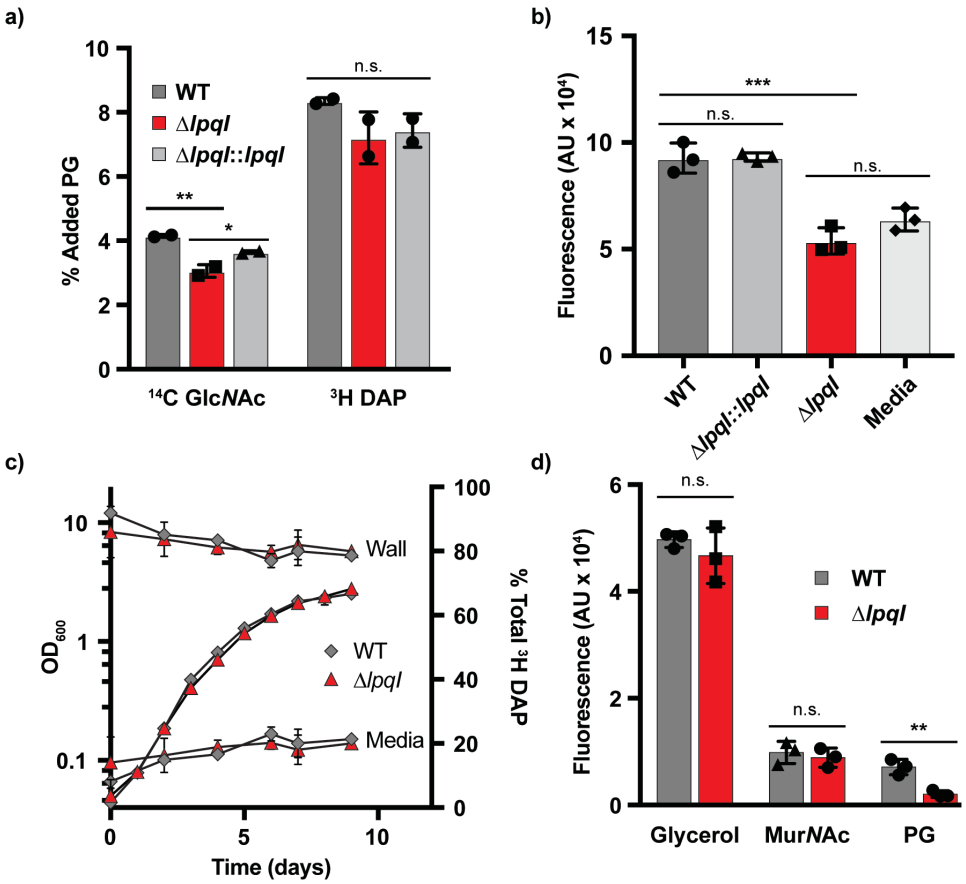

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585 **Figure 3. *M. bovis* BCG is able to recycle PG. a)** *M. bovis* BCG WT, Δ*lpqI* and Δ*lpqI*::*lpqI*
586 were incubated with 30,000 CPM of ^{14}C Glc*N*Ac-labelled muropeptides or 100, 000 CPM of
587 ^{3}H DAP-labelled muropeptides for 10 days after which the cell wall material was isolated and
588 subjected to liquid scintillation counting (n = 2). **b)** The same strains were incubated with 1
589 mM 4MU-Glc*N*Ac in minimal media. After 3 days the fluorescence of the cultures were
590 measured (n = 3). **c)** *M. bovis* BCG WT and Δ*lpqI* were simultaneously evaluated for release of
591 cell wall peptides and growth (n = 3). **d)** The same strains were evaluated for their growth using
592 glycerol, Mur*N*Ac and PG as sole carbon sources using a resazurin assay (n = 3; *** = $p < 0.001$; ** = $p < 0.005$).

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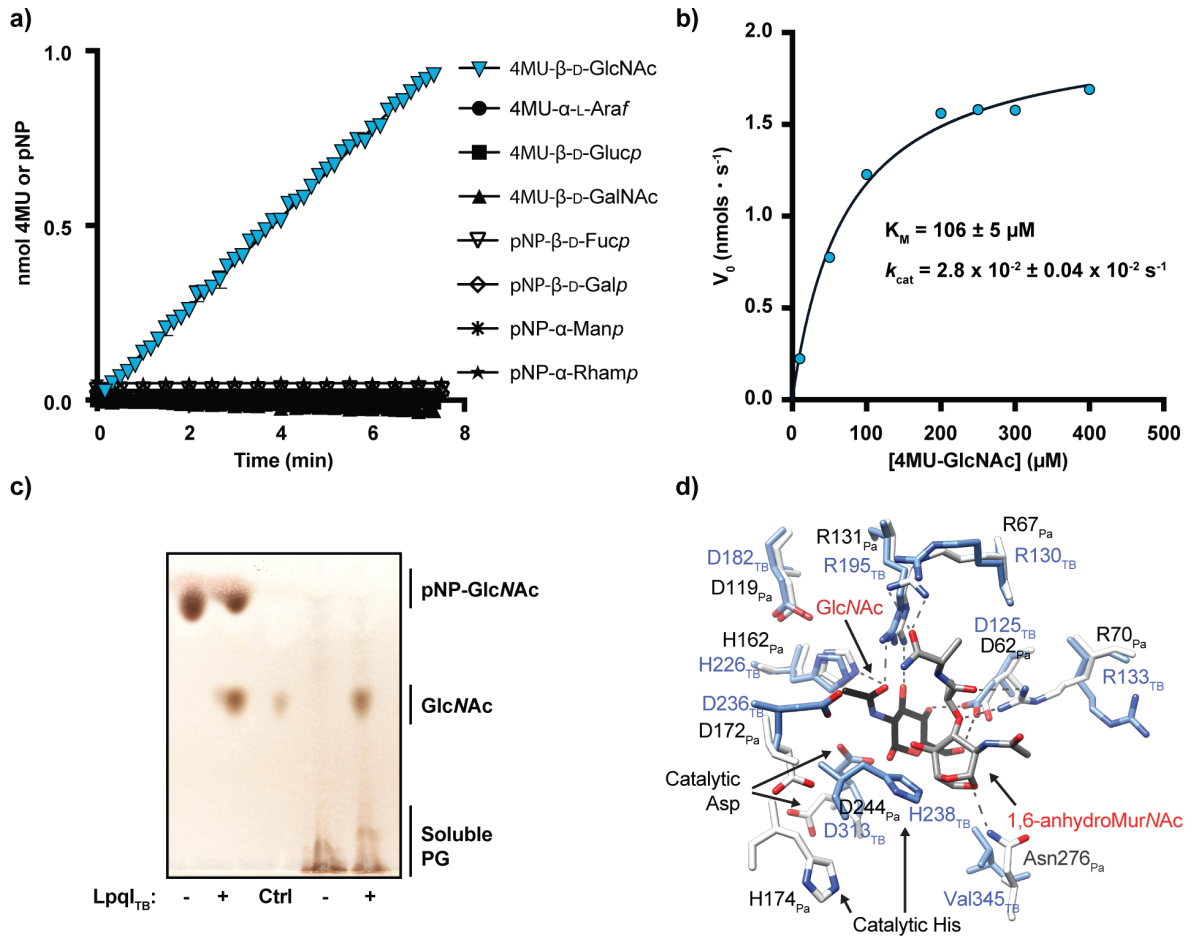

Figure 4. LpqI is an authentic NagZ-type enzyme. a) Reactions including 1 µM LpqI and the indicated chromogenic substrates at 1 mM were incubated at 37 °C and release of pNP or 4MU was followed by absorbance or fluorescence respectively. **b)** LpqI was incubated with increasing concentrations of 4MU-Glc*N*Ac. The rate of 4MU release was plotted and the curve fit with the Michaelis-Menton equation using GraphPad Prism 7.0. (n = 3) **c)** LpqI is able to release Glc*N*Ac from soluble muropeptides derived from *M. smegmatis* mc^{2}155 PG. **d)** The active site of LpqI is highly conserved as evidenced by the nearly identical positioning of key binding residues observed in the Glc*N*Ac, 1,6-anhydroMur*N*Ac complex with $NagZ_{Pa}$ (PDB: 5G3R) with an overall RMSD of 1.01 Å.

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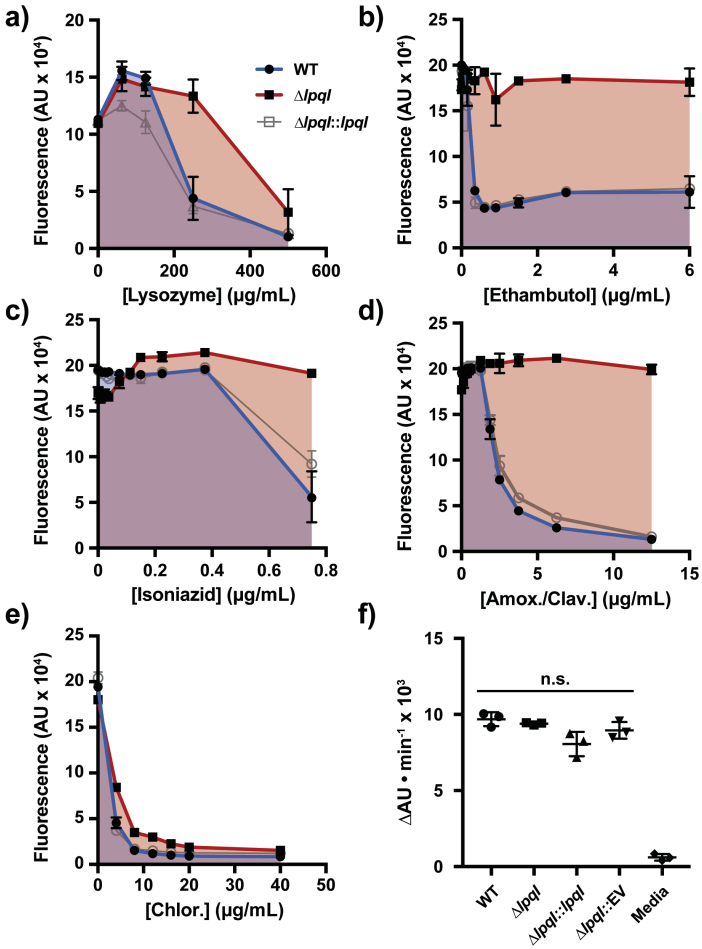

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611 **Figure 5. Loss of LpqI leads to lysozyme and antibiotic resistance. a-e)** *M. bovis* BCG WT,
612 Δ*lpqI* and Δ*lpqI*::*lpqI* were incubated with increasing concentrations of lysozyme or antibiotics
613 at a starting OD_{600} of 0.1. After 7 days incubation total growth was assessed using a resazurin
614 assay, where total fluorescence correlates with respiration and growth (n = 3). f) *M. bovis* BCG
615 WT, Δ*lpqI* and Δ*lpqI*::*lpqI* and Δ*lpqI*::EV were incubated with EtBr and the rate of EtBr was
616 monitored as an increase in fluorescence. No significant differences were found in pairwise t-
617 tests across all strains (n = 3).
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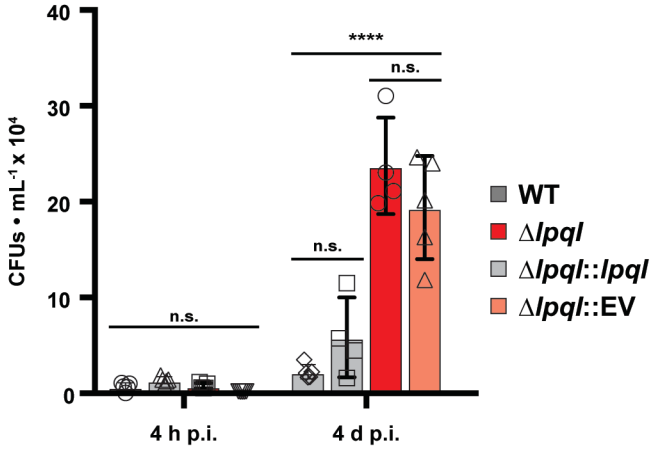

Figure 6. Loss of *lpqI* leads to increased growth in BMDMs. Freshly prepared BMDs were infected with the indicated strains at a multiplicity of infection of 2 and incubated for at 37 °C. At the indicated times the macrophages were lysed with saponin and CFUs were measured on 7H11 agar after 3 weeks incubation ($n \geq 4$; **** = $p < 0.0001$).

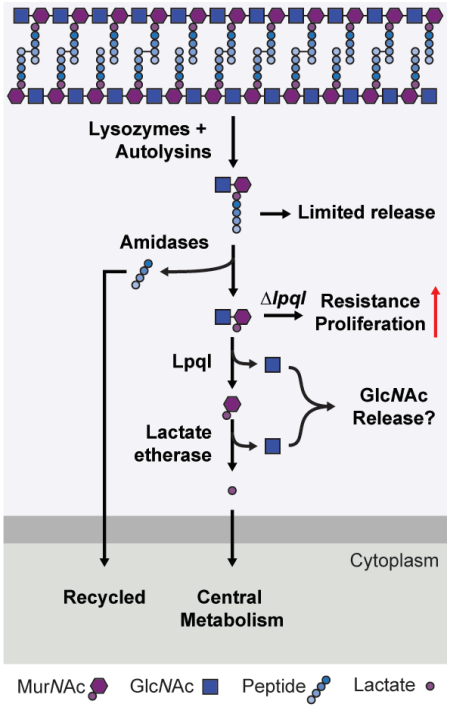

Figure 7. Peptidoglycan recovery pathway in pathogenic mycobacteria. Based on our observations we can propose the following model for PG recycling and recovery in mycobacteria. Cleavage of the cell wall by endogenous autolysins or host-derived lysozyme generates muropeptides. These are subsequently degraded by amidases. LpqI then cleaves the disaccharide which is followed by D-lactyl-ether cleavage. Lactate can then be used by the cell under aerobic conditions and Glc*N*Ac (or its derivatives) are released. Perturbation of this system by inhibiting LpqI leads to increased resistance to anti-mycobacterial agents as well as increased proliferation in the host.

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