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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

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

Introduction

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

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

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

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

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

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

Materials and methods

Bacterial strains and growth conditions:

M. bovis BCG (Pasteur) and related mutants were maintained on Middlebrook 7H10 agar or 7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween 80. Where appropriate kanamycin or hygromycin was added at 25 or 50 μg•mL^{-1}, respectively. *M. smegmatis* mc^{2}155 were maintained on Tryptic Soy Broth or Tryptic Soy Agar where appropriate. For growth on defined carbon sources, strains were grown in Sauton's minimal 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 ammonium citrate, 0.05% tyloxopol) containing either 5 mM of each sole-carbon unless stated otherwise[15]. *Escherichia coli* strains were grown in lysogeny broth and supplemented with kanamycin at 50 μg•mL^{-1} or hygromycin at 150 μg•mL^{-1} where appropriate.

Mutant generation

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

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

Antimicrobial testing

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

Rapid purification of mAGP

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

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

Large-scale purification of PG

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

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

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

Synthesis of 4MU-D-lactate

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

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

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

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

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

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

Cloning and purification of Rv0237

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

Crystallography

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

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

Kinetic characterisation of Rv0237

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

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

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

Infection of bone marrow-derived macrophages

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

Results

Peptidoglycan Recycling Genes in *Mycobacteria*

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

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

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

Utilisation of peptidoglycan components by mycobacteria

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

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

Mechanism of Mur*N*Ac metabolism

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

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

Uptake of PG metabolites by mycobacteria

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

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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

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

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

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

Characterisation of a Δ*lpqI* mutant

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

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

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

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

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

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

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

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Discussion

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

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

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

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

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

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

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

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

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

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

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547 **Author Contributions**

548 Conceived of the study: P.J.M. Conceived and designed the experiments: P.J.M., G.V.M., M.S.,
549 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
550 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
551 the paper: P.J.M, G.V.M., M.S., A.L.L., G.S.B.

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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)

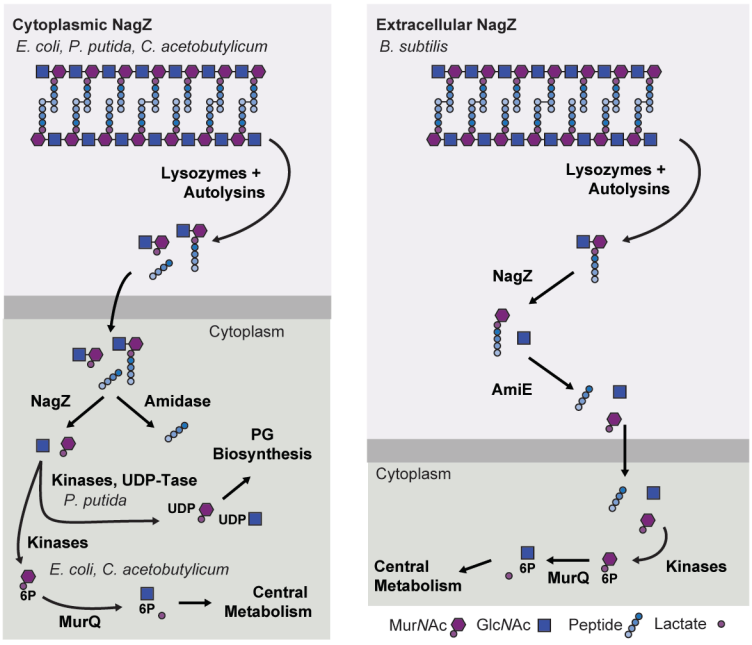

Figure 1. Overview of PG-recycling. a) The genes involved in PG-recycling fall into functional groups that are typically conserved amongst closely related species, with two major Mur*N*Ac recovery systems so far identified (AnmK/MurQ and AngK/MurU). **b)** For a complete listing of gene conservation 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.

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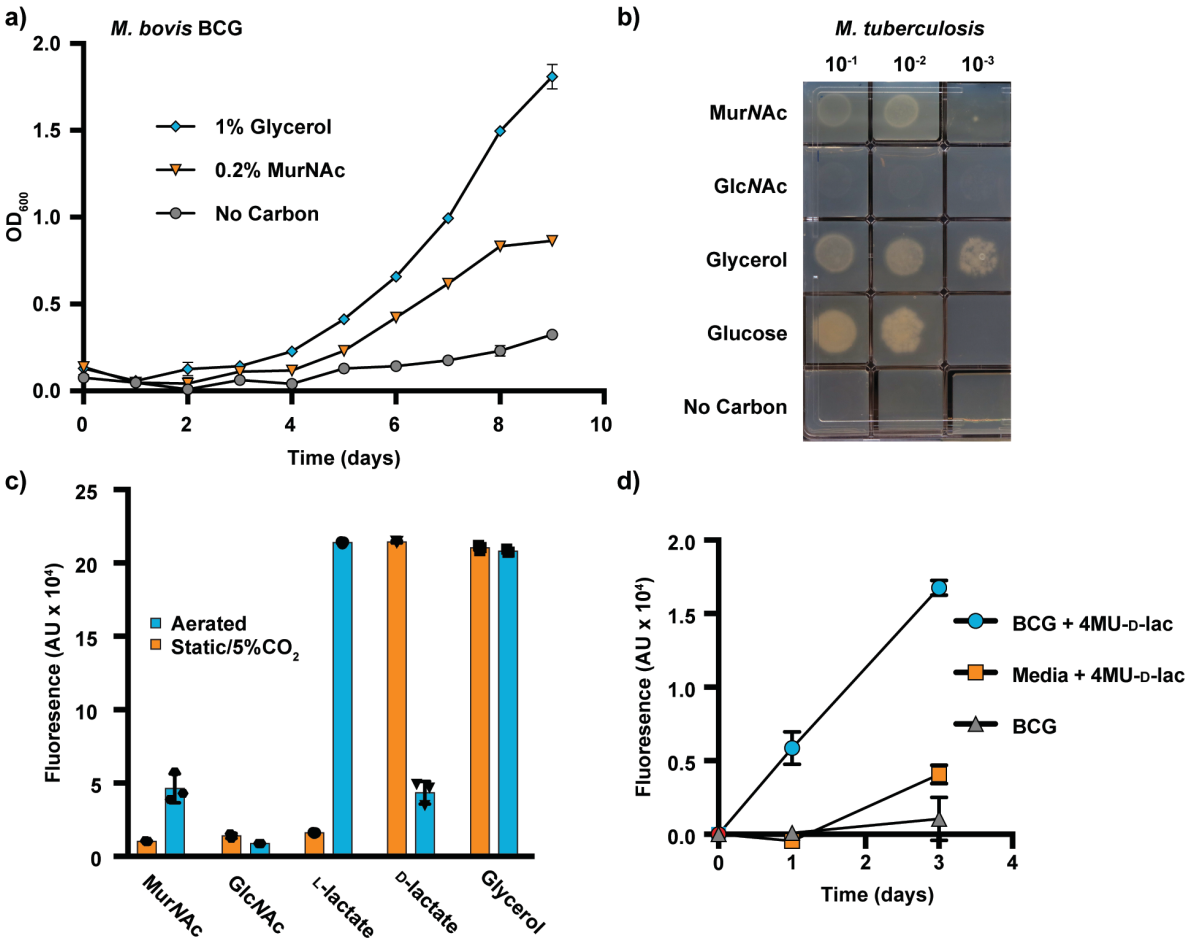

Figure 2. *M. tuberculosis* and *M. bovis* BCG are able to recycle Mur*N*Ac. a) *M. bovis* BCG WT was inoculated at a starting OD_{600} of 0.1 in minimal media containing glycerol and tyloxopol, Mur*N*Ac or no carbon and growth was monitored daily by taking OD_{600} readings at the indicated concentrations (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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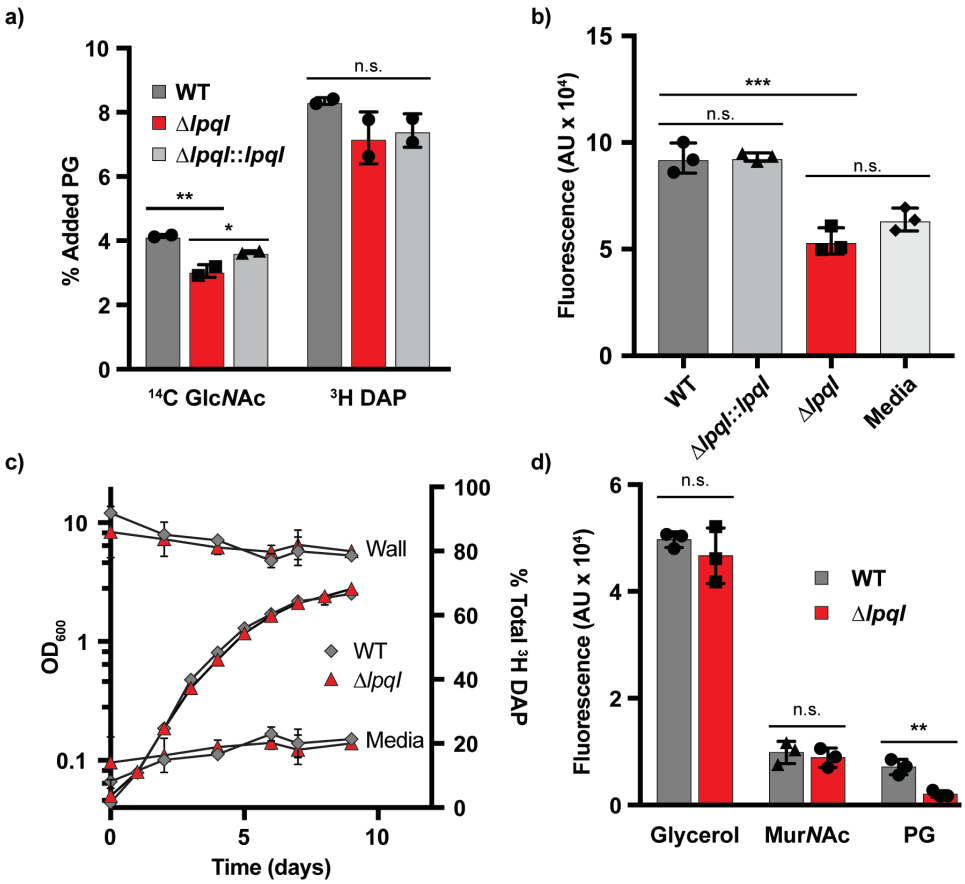

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585 **Figure 3. *M. bovis* BCG is able to recycle PG. a)** *M. bovis* BCG WT, Δ*lpqI* and Δ*lpqI*::*lpqI*
586 were incubated with 30,000 CPM of ^{14}C Glc*N*Ac-labelled muropeptides or 100, 000 CPM of
587 ^{3}H DAP-labelled muropeptides for 10 days 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; *** = $p < 0.001$; ** = $p < 0.005$).
593
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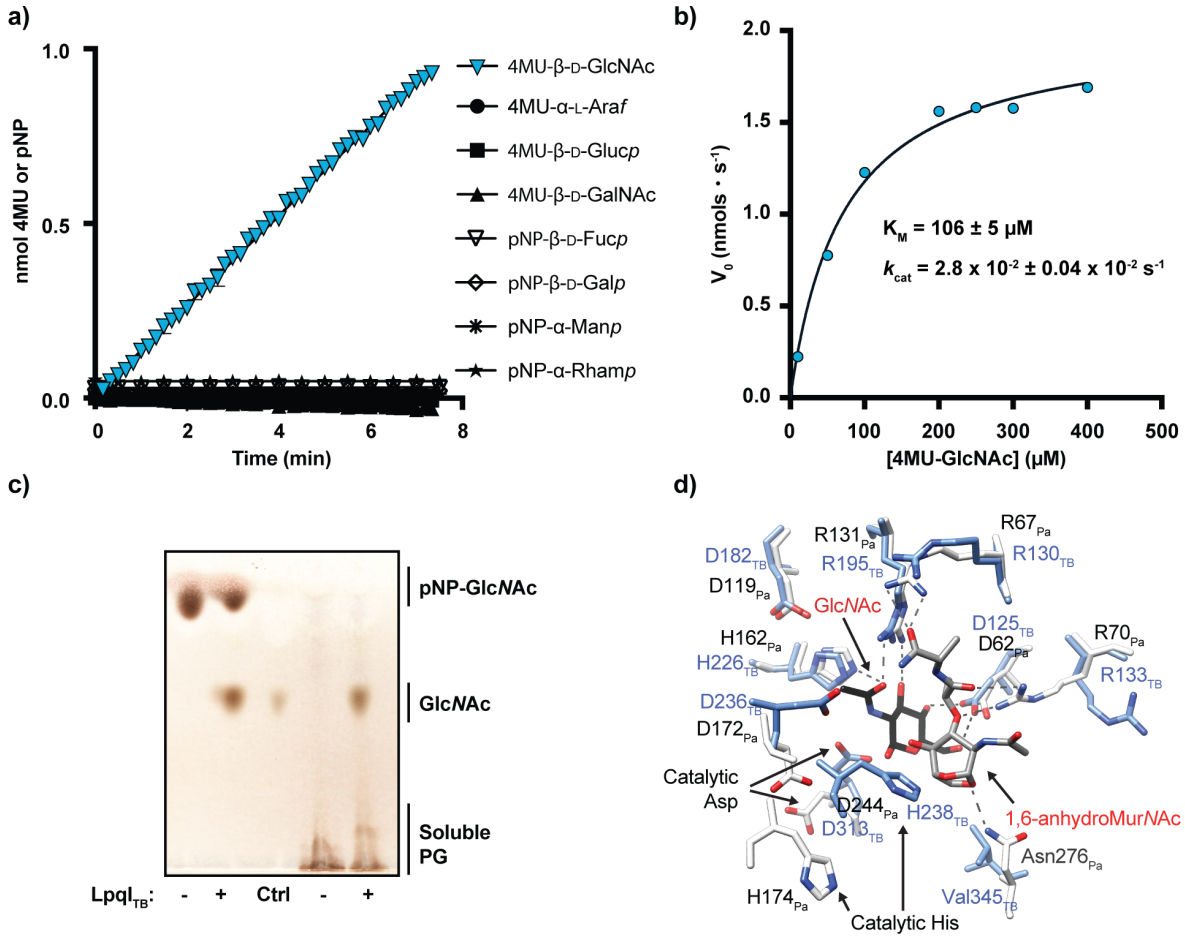

Figure 4. LpqI is an authentic NagZ-type enzyme. a) Reactions including 1 µM LpqI and the indicated chromogenic substrates at 1 mM were incubated at 37 °C and release of pNP or 4MU was followed by absorbance or fluorescence respectively. **b)** LpqI was incubated with increasing concentrations of 4MU-Glc*N*Ac. 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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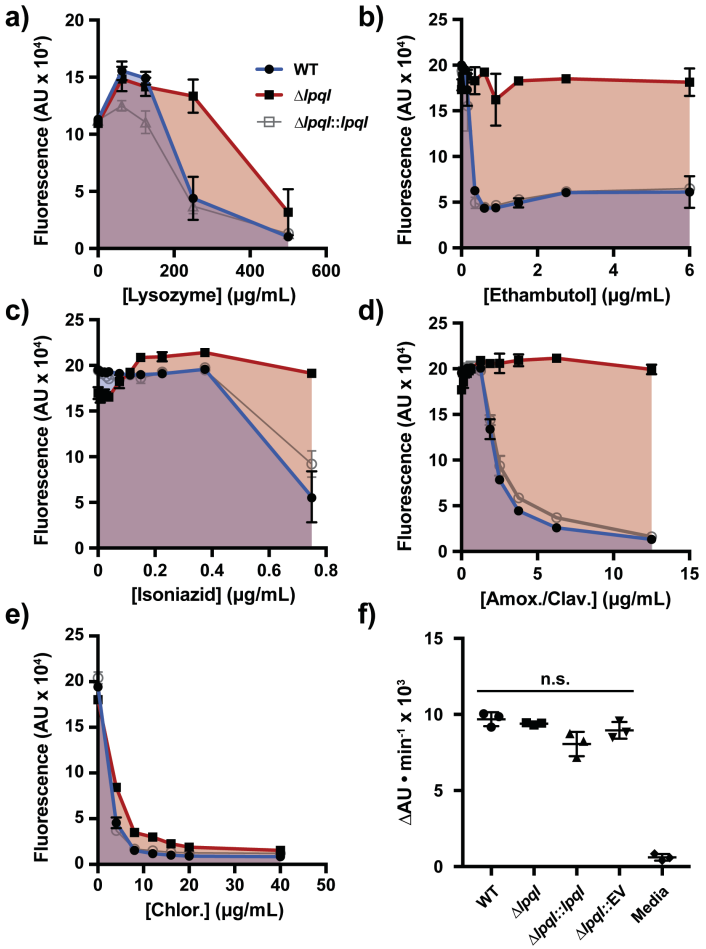

Figure 5. Loss of LpqI leads to lysozyme and antibiotic resistance. a-e) *M. bovis* BCG WT, Δ*lpqI* and Δ*lpqI*::*lpqI* were incubated with increasing concentrations of lysozyme or antibiotics at a starting OD_{600} of 0.1. After 7 days incubation total growth was assessed using a resazurin assay, where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

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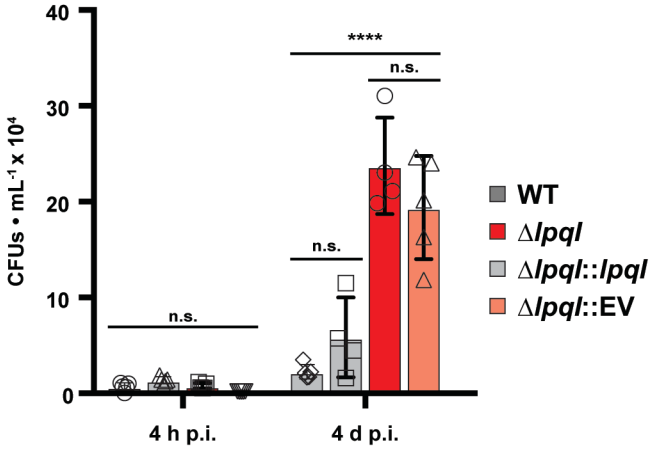

Figure 6. Loss of *lpqI* leads to increased growth in BMDMs. Freshly prepared BMDs were infected with the indicated strains at a multiplicity of infection of 2 and incubated for at 37 °C. At the indicated times the macrophages were lysed with saponin and CFUs were measured on 7H11 agar after 3 weeks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

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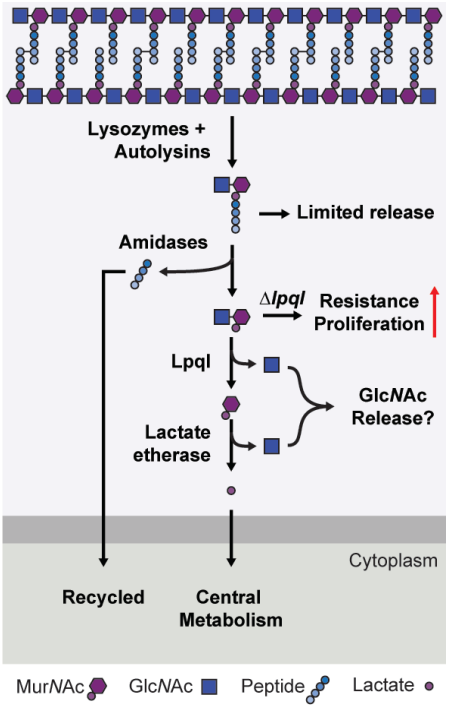

Figure 7. Peptidoglycan recovery pathway in pathogenic mycobacteria. Based on our observations we can propose the following model for PG recycling and recovery in mycobacteria. Cleavage of the cell wall by endogenous autolysins or host-derived lysozyme generates muropeptides. These are subsequently degraded 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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