The hydrolase LpqI primes mycobacterial peptidoglycan recycling

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Growth and division by most bacteria requires remodelling and cleavage of their cell wall. A byproduct of this process is the generation of free peptidoglycan (PG) fragments known as muropeptides, which are recycled in many model organisms. Bacteria and hosts can harness the unique nature of muropeptides as a signal for cell wall damage and infection, respectively. 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 able to recycle components of their PG. We demonstrate that the core mycobacterial gene lpqI, encodes an authentic NagZ β-N-acetylglucosaminidase and that it is essential for PG-derived amino sugar recycling via an unusual pathway. Together these data provide a critical first step in understanding how mycobacteria recycle their peptidoglycan.
The cell wall of *Mycobacterium tuberculosis* is built upon a foundation of peptidoglycan (PG). The remainder of this structure is formed by the modification of muramic acid residues with an arabinogalactan polymer that in turn is esterified by mycolic acids. This waxy coating not only contributes to drug resistance in *M. tuberculosis*, but is also the target of several mycobacteria-specific antibiotics. 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 in treating these drug-resistant infections. Despite their therapeutic promise, we know relatively little about the turnover of PG in mycobacteria, which is the eventual target of β-lactam antibiotics.

For most bacteria, maintenance of a PG sacculus is an essential aspect of life. PG is a heteropolymer comprised of glycan chains with a repeating disaccharide motif of N-acetylmuramyl pentapeptide (GlcNAc-MurNAc), which are then cross-linked to one another via short peptides attached to the C-3 d-lactyl moiety of MurNAc (Fig. 1a). The integrity of this macromolecule must be maintained under most growth conditions and its rupture leads to lysis and cell death. 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. 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 fragments.

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-anhydroMurNAc products. 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. 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. In many organisms, soluble PG acts as a potent immune stimulator once sensed by NOD receptors and other pattern recognition receptors.

Aside from host organisms, PG metabolites are also important signalling molecules for the bacteria themselves. 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. The recycling pathway typically involves the step-wise degradation of the polymer into its monomeric constituents, monosaccharides, and amino acids (Fig. 1b). Despite common biochemical steps, compartmentalisation of these steps tends to be organism specific. 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 (Fig. 1b). At the same time, the stem peptides are degraded to smaller components and typically shunted back into PG biogenesis. Growth on MurNAc as a sole carbon source has never been demonstrated for a bacterium that lacks MurQ. Furthermore, recycling of MurNAc in a bacterium that lacks MurQ has only been described in *Pseudomonas putida*, and many bacteria, including mycobacteria, are not thought to recycle their PG at all.

In the present study, we sought to determine whether mycobacteria are capable of recycling their PG and if so, what impact this has on the bacterium. We demonstrate that these bacteria do indeed possess the biochemical capacity to recycle PG elements and determined how the bacteria generate free MurNAc for recycling. Our data indicate that loss of a key recycling enzyme, LpqI, leads to increased antibiotic and lysozyme resistance.

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**Fig. 1** Overview of PG recycling. **a** The basic building block of PG is GlcNAc-MurNAc-pentapeptide. Enzymes produced by the bacterium or the host are able to cleave every major linkage in PG. **b** Known PG-recycling machinery is variable with respect to the localisation of NagZ and the subsequent conversion to GlcNAc-1P or UDP-GlcNAc/MurNAc. All known MurNAc recovery systems that sustain bacterial growth (as opposed to strictly recycling e.g. *P. putida*) terminate at MurQ in the cytoplasm.
Results
PG-recycling genes in mycobacteria. The genome of *M. tuberculosis* encodes many lytic enzymes, including at least five resuscitation-promoting factors (Rpfs) and > 10 peptidases and amidases in addition to penicillin-binding proteins with potential lytic activities16. The Rpfs are most likely lytic transglycosylases with the product of RpfB having been recently confirmed as a GlcNAc-1,6-anhydroMurNAc disaccharide-peptide27. Although *M. tuberculosis* does appear to encode at least one lysozyme, Rv2525, its activity has not been demonstrated18. A recent comparative study of PG-active enzymes in mycobacteria indicated that although significant differences exist, enzymes that can likely degrade all of the major covalent linkages of PG are encoded in the genomes of all mycobacteria16. The products of most of these enzymes have not been experimentally demonstrated, however, their conservation underscores the importance of PG-remodelling 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 vitro19,20. Given the slow release of PG fragments by mycobacteria, we evaluated the presence of known PG-recycling systems in the genome of several corynebacterial species (Supplementary Table 2)15,21. BLAST analysis of the *Corynebacterium glutamicum*, *M. tuberculosis*, *Mycobacterium leprae*, and *M. 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 characterised as glucose-kinases although they have not been directly tested for amino sugar-phosphotransferase activity22. This contrasts sharply with *M. smegmatis* for which an apparently complete “classical” muropeptide recovery system exists, making it a poor model for the PG metabolism of *M. tuberculosis* (Supplementary Table 2). Taken together, the available data indicates that *M. tuberculosis* is almost all other mycobacteria lack most of the known PG-recycling genes from other bacteria, with only two conserved genes potentially associated with PG-recycling, (nagA—Rv3332, nagZ/lpqI—Rv0237).

Biochemical and structural characterisation of LpqI. In previously characterised PG-recycling systems free amino sugars are produced by NagZ, which belongs to the CAZy glycosidase hydrolase family 3 (GH3)23. This family is a large group of enzymes that have hydrolytic and phosphorylating activity and remove β-linked sugars from proteins and polysaccharides24,25. The β-N-acetylglucosaminidase sub-family including all known NagZ enzymes utilise a conserved Asp-His catalytic dyad, which has been well characterised26,27. A BLAST search of the *M. tuberculosis* H37Rv genome revealed only one NagZ ortholog, which was previously named LpqI in light of its identification as a lipoprotein including an appropriately positioned lipobox at the N-terminus of the protein26. As a lipoprotein LpqI is expected to be found attached to the external face of the cytoplasmic membrane, which is consistent with proteomics results28. LpqI has also been identified as a likely mannosylated glycoprotein in a proteomics screen using ConA chromatography29. The LpqI gene is found in all mycobacteria with sequenced genomes including *M. leprae*, which has a substantially reduced genome suggesting that it is involved in a conserved process across all mycobacteria (Supplementary Table 2, Supplementary Fig. 1).

Given the absence of other PG-recycling-associated genes, we sought to identify the function of LpqI. Although LpqI bears significant sequence similarity to known β-N-acetylglucosaminidases, recent studies have demonstrated that divergent activities for this sub-family of enzymes are possible25. These activities included the ability to release sugars other than GlcNAc from reporter substrates and apparent phosphorolytic activity. To test this, we cloned, expressed and purified LpqITB using an N-terminal His6-SUMO tag, which was subsequently cleaved from the protein. We first determined whether the protein was in fact a β-N-acetylglucosaminidase by testing its activity on a variety of substrates including many sugars that would be found in the cell wall of mycobacteria. Using convenient reporter sugars we assessed the ability for the enzyme to release β-nitrophenololate or 4-methylumbelliferone from a variety of conjugated sugars (Fig. 2a). Although not exhaustive, this set of sugars covers most major modifications to the cell wall including the AG itself, O-mannose modifications of proteins, GalN modification of arabianin, the rhamnose-linker sugar of AG and the GlcNAc and GlcN found in PG. The only detectable activity for LpqI was with GlcNAc-containing substrates (Fig. 2a). Critically, in mycobacteria, this sugar is limited to the backbone of PG and a small amount in the linker unit (MurNAc-6-P-Rha-GlcNAc-galactan) between PG and arabinogalactan. The Michaelis‒Menten constants (\(K_m=2.0 \times 10^{-2} \pm 0.04 \times 10^{-2} \mu M\) and \(K_m=72 \pm 5 \mu M\)) of LpqI using 4MU-GlcNAc as a substrate were found to be similar to other NagZ enzymes using this substrate (Fig. 2b)26. In a similar assay we were also able to show that LpqI releases GlcNAc from soluble PG fragments (Fig. 2c), including PG fragments with a 1,6-anhydroMurNAc terminus (Supplementary Fig. 2). Although 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 phosphorylases25. Another GH3 β-N-acetylglucosaminidase was recently reported to lack this latter activity, suggesting that it may not be a general property of the family30. We tested the activity of the enzyme under the same conditions as reported previously (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, 5 mM MgSO4, 10% glycerol, 1 mM DTT) 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 GlcNAc on TLCs and not GlcNAc-1-P (Supplementary Fig. 3).

To confirm that LpqI is an exo-acting enzyme rather than an autolytic β-N-acetylglucosaminidase we evaluated the activity of the enzyme using defined-length chitooligosaccharides. As shown in Supplementary Fig. 4a, LpqI produces GlcNAc5 and GlcNAc from GlcNAc6. This is in contrast to the known endo-acting chitinase ChitB, which produces GlcNAc2 and GlcNAc4 from the same substrate31. We further tested the ability of the enzyme to act as an autolysin and release soluble PG from insoluble material by incubating the enzyme with fluorescently labelled PG. In this assay, LpqI was not observed to release any soluble PG in contrast to the muramidase mutanolysin (Supplementary Fig. 4b). Together these data support the role of LpqI as an exo-acting enzyme involved in fragment recycling, rather than an endo-acting autolysin.

To further confirm its function and validate its role in PG-recycling, we solved the 1.96 Å X-ray crystal structure of LpqI (PDB code: 6GFV; Supplementary Fig. 1, Supplementary Table 3). 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 (Supplementary Fig. 5). Alignment of LpqI with the NagZ/GlcNAc1,6-anhydroMurNAc complex from *P. aeruginosa* (NagZ/pul; PDB:5G3R) or NagZ from *B. subtilis* (PDB:4GYJ) using the SSM Superpose function in COOT resulted in a root-mean-square deviation of 2.30 Å and 1.63 Å, respectively (Supplementary Fig. 5). Superposition of the post-cleavage

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NagZPa complex with LpqI indicates that the appropriate coordinating residues for MurNAC or 1,6-anhydroMurNAC recognition are intact in LpqI, supporting its role in PG-recycling (Fig. 2d).

LpqI-catalysed utilisation of PG components by mycobacteria. Having confirmed the in vitro activity of LpqI, we sought to determine the fate of its reaction products, MurNAC and GlcNAC, in growing M. bovis BCG. Prior research has shown that most mycobacteria are unable to use GlcNAC as a sole carbon source, with M. smegmatis being one of the notable exceptions. Amino acids including d-Ala and d-glu have previously been shown to serve as nitrogen sources for M. tuberculosis, but they do not serve as carbon sources. 1-Ala has also been shown to not serve as a carbon source for M. bovis. To our knowledge, recycling of GlcNAC or MurNAC by mycobacteria has not been reported, nor has recycling been tested for soluble PG fragments. To evaluate this, M. bovis BCG was cultured in minimal media supplemented with glycerol (1% v/v) or MurNAC (0.2% w/v) in Sauton’s minimal media with constant aeration. As observed in Fig. 3a, M. bovis BCG was able to grow using MurNAC as a sole carbon source. 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 (Fig. 3b). Intriguingly growth on MurNAC in broth was heavily dependent on the aeration of the culture. In contrast, growth on glycerol was unaffected by this change (Fig. 3c). To further evaluate the potential for mycobacteria to take up GlcNAC but use it for purposes other than central metabolism, we tested the ability of M. bovis BCG to incorporate 14C GlcNAC into whole cells. Under different growth conditions (rich medium, carbon-poor medium, aerated cultures, static cultures) we were unable to detect significant amounts of GlcNAC being taken up by M. bovis BCG. In all cases, the c.p.m. of the label in whole cells was less than or equal to unlabelled controls. We conclude from these data that pathogenic mycobacteria are able to utilise MurNAC, but not GlcNAC in an O2-dependent fashion.

Mechanism of MurNAC metabolism. Given its structural similarity to GlcNAC, the ability of M. tuberculosis and M. bovis BCG to grow on MurNAC was surprising and so we evaluated the biochemical processing steps associated with MurNAC utilisation. MurNAC is a combination of GlcNAC and d-lactate joined by an ether linkage. This suggests that the bacterium is either using the GlcNAC moiety for glycolysis, or shunting the lactate derived from MurNAC into the TCA cycle. We tested this by inhibiting glycolysis with 2-deoxyglucose in cultures grown using MurNAC, glucose, and glycerol as sole carbon sources (Supplementary Fig. 6). These data suggested that the pathway of MurNAC utilisation did not require glycolysis and indicated that the lactate

Fig. 2 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. Cleavage of 4MU-GlcNAC is indicated by blue triangles. b LpqI was incubated with increasing concentrations of 4MU-GlcNAC. The rate of 4MU release was plotted and the curve fit with the Michaelis–Menten equation using GraphPad Prism 7.0. (n = 3, error bars are ± SD). c Thin-layer chromatograph of reaction products showing that LpqI is able to release GlcNAC from soluble muropeptides derived from M. smegmatis mc2155 PG. d The active site of LpqI is highly conserved as evidenced by the similar positioning of key binding residues observed in the GlcNAC, 1,6-anhydroMurNAC complex with NagZPa (PDB: 5G3R) the main chains of which have been aligned with an RMSD of 2.3 Å using LSQ KAB.
moiety of MurNAc, was instead most likely serving as a carbon source. Consistent with this, when used as a sole carbon source, growth on \(\alpha\)-lactate and MurNAc was O\(_2\)-dependent, whereas \(\beta\)-lactate was better utilised under static, 5% CO\(_2\) culture conditions, where MurNAc could not be used as a carbon source (Fig. 3c).

These data allow us to hypothesise a mechanism by which \(M. bovis\) BCG metabolises MurNAC. Given that metabolism of \(\alpha\)-lactate and MurNAc are O\(_2\)-dependent, we anticipate that use of MurNAC follows cleavage of the \(\beta\)-lactate from MurNAc to produce lactate and GlcNAC. In this case, the O\(_2\) dependency on MurNAC metabolism is likely the result of an O\(_2\)-dependent lactate dehydrogenase. Consistent with this, two O\(_2\)-dependent \(\alpha\)-lactate dehydrogenases have been identified in \(M. tuberculosis\) (Rv0694, Rv1872c), which are required for growth on L-lactate as a sole carbon source\(^36\).

Given the unusual nature of MurNAC, generation of free lactate by the bacterium would require the activity of a specific lactyl-etherase. To test for the presence of this activity in whole cells, we synthesised a 4MU-\(\alpha\)-lactate derivative for use in a reporter-analogue of MurNAC (Supplementary Fig. 7). 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 (Fig. 3d). Together these data support a model where mycobacteria cleave the lactyl moiety from MurNAC by an as-yet unidentified enzyme and utilise the product of that reaction as a carbon source under aerated conditions.

**LpqI-dependent uptake of PG metabolites by mycobacteria.**

Although our data strongly support metabolism of MurNAC by \(M. bovis\) BCG, we wanted to confirm the role of LpqI in mycobacterial PG-recycling. To do this we constructed a mutant strain of \(M. bovis\) BCG-lacking \(lpqI\) using specialised transduction\(^37\). To validate that LpqI is the only \(\beta\)-N-acetyl glucosaminidase produced by \(M. bovis\) BCG, we used a whole-cell \(\beta\)-N-acetyl glucosaminidase assay. This demonstrated that \(M. bovis\) BCG \(\Delta lpqI\) is devoid of \(\beta\)-N-acetyl glucosaminidase activity as the amount of 4MU released is not significantly different from the spontaneous release in sterile media (Fig. 4a). This deficiency is complemented by re-introduction of the \(lpqI\) gene at a distal chromosomal location under the control of its native promoter (\(\Delta lpqI::lpqI\)) and is not complemented by the empty vector (\(\Delta lpqI::EV\)) (Fig. 4a). Growth of \(\Delta lpqI\) in vitro is also unaltered as compared to the wild-type (Fig. 4b). This mutant therefore provided us with an opportunity to probe the role of disaccharide cleavage in mycobacterial PG-recycling.

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 assessed the impact of the loss of \( \text{lpqI} \) on the recycling of cell wall material. To investigate this, we generated radio-labelled muropeptides and tested them in whole-cell uptake assays. Radio-labelled muropeptides had to be generated in \( \text{M. smegmatis} \) owing to the inability of \( \text{M. bovis} \) BCG to take up radiolabeled GlcNAc as a sole carbon source\(^3\). This, along with the absence of the muropeptides with \( \text{lpqI} \) (Fig. 4c). Together these data indicate that \( \text{lpqI} \) is required for PG-derived amino sugar recycling in \( \text{M. bovis} \) BCG.

**Phenotypic characterisation of a \( \Delta \text{lpqI} \) mutant.** As indicated above, loss of \( \text{lpqI} \) did not alter the growth rate of the bacterium in vitro. However, given that NagZ-like proteins have been found to play a role in \( \beta \)-lactam sensitivity in other bacteria we sought to determine the antimicrobial sensitivity of the \( \Delta \text{lpqI} \) strain. In contrast to inhibition of \( \text{P. aeruginosa} \) NagZ, deletion of \( \text{lpqI} \) resulted in an increase in survival in the presence of lysozyme and all cell wall active antibiotics tested (Fig. 5a–d)\(^3\). A smaller impact on survival in the presence of the protein synthesis inhibitor chloramphenicol was observed (Fig. 5e). This increase in resistance is not owing to a change in cell wall permeability as determined by ethidium bromide uptake (Fig. 5f). These data indicate that \( \text{lpqI} \)-dependent amino sugar recycling is involved in the expression of antibiotic and lysozyme resistance by mycobacteria in vitro.

**Discussion**

In an attempt to develop diagnostic media for the identification of mycobacteria, several groups in the 1960s observed that \( \text{M. tuberculosis} \) and most other mycobacteria could not metabolise GlcNAc as a sole carbon source\(^33,34\). This, along with the absence of

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**Fig. 4** \( \text{M. bovis} \) BCG is able to recycle PG. a. \( \text{M. bovis} \) BCG WT, \( \Delta \text{lpqI} \), \( \Delta \text{lpqI}::\text{Empty Vector} \) were incubated with 1 mM 4MU-GlcNAc in minimal media. After 3 days the fluorescence of the cultures were measured \( (n = 3; \text{error bars} = \pm \text{SD}) \). b. \( \text{M. bovis} \) BCG WT (grey diamonds) and \( \Delta \text{lpqI} \) (red triangles) were simultaneously evaluated for release of cell wall peptides and growth \( (n = 3; \text{error bars} = \pm \text{SD}) \). c. \( \text{M. bovis} \) BCG WT (dark grey bars), \( \Delta \text{lpqI} \) (red bars), and \( \Delta \text{lpqI}::\text{lpqI} \) (light grey bars) were evaluated for their growth using MurNAc (5 mM), GlcNAc-MurNAc (2 mM), soluble PG from \( \text{M. smegmatis} \) mc\(^2\)155 wild-type or \( \Delta \text{namH} \) (5 and 2.5 mM, respectively), and soluble PG, which had been pre-digested with LpqI (striped bars) as sole carbon sources using a resazurin assay \( (n = 3; *** = p < 0.001; ** = p < 0.005) \). Statistical significance determined using a two-tailed \( t \)-test in GraphPad Prism 7.

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**Discussion**

In an attempt to develop diagnostic media for the identification of mycobacteria, several groups in the 1960s observed that \( \text{M. tuberculosis} \) and most other mycobacteria could not metabolise GlcNAc as a sole carbon source\(^33,34\). This, along with the absence of...
of known PG recycling-associated genes lead to the assumption that PG recycling is absent in pathogenic mycobacteria. Based on our data and the literature, it is clear that not only is M. tuberculosis able to recycle its PG, it is generating two distinct classes of molecules. These are GlcNAc-MurNAC-peptide, which is sensed by the host, and GlcNAC-MurNAC that is sensed by the bacterium. Although host-sensing of PG is unaffected by the presence of GlcNAC on NOD-stimulatory molecules, our data indicate that LpqI acts as a regulator for GlcNAC-MurNAC levels by cleaving disaccharides and allowing the breakdown of MurNAC. In other bacteria, cell wall damage can trigger various stress responses, and so it is likely that a build-up of GlcNAC-MurNAC disaccharides may trigger a stress-like response in mycobacteria. Consistent with this, lpqI is encoded adjacent to a universal stress response protein in several species of mycobacteria (Supplementary Fig. 1). As a starting point to investigate PG recycling in M. tuberculosis, we characterised the core mycobacterial protein, LpqI. Despite the absence of other known PG-recycling proteins, we have shown that LpqI is an authentic exo-acting β-N-acetylgalactosaminidase, which is able to cleave PG fragments in vitro. Consistent with a role in PG-recycling, M. bovis BCG ΔlpqI is unable to grow on soluble PG as a sole carbon source, whereas recycling of the stem-peptide is unaltered in this mutant. Together, our data demonstrate that M. bovis BCG and M. tuberculosis remove the stem-peptide from PG fragments prior to disaccharide cleavage and lactyl-ether removal (Fig. 6).
processing of GlcNAc-MurNac by LpqI prior to lactyl-ether cleavage is also supported by our LpqI crystal structure in which the lactate-binding residue R67 from the structure is conserved (LpqI: R130), suggesting that the physiological substrate of this enzyme possesses the lactyl group.32

Our sole carbon source assays indicate that although the bacteria are unable to metabolise GlcNAc, surprisingly, they can use MurNac as a sole carbon source (Fig. 3). This is despite the fact that they lack an ortholog of the only known lactyl-etherase, MurQ that cleaves an otherwise stable lactyl-ether in the cytoplasm of most model organisms (Fig. 1). Our data indicate that rather than using the GlcNAc portion of the MurNac, the bacteria are cleaving the lactyl-ether and metabolising the liberated lactate. During our study we found that M. bovis BCG was only able to grow on MurNac under aerated conditions. This was also found to be the case for L- but not D-lactate, which served as a carbon source under anaerobic conditions and GlcNAc (or its derivatives) are most likely released. Perturbation of this system by deleting LpqI leads to increased resistance to anti-mycobacterial agents.

In conclusion, we have identified a PG recovery pathway in pathogenic mycobacteria. We have shown that this occurs in a step-wise fashion by removing the PG disaccharide, lactyl-ether from the free MurNac. Finally, we have shown that recycling of PG by these bacteria is important for lysozyme and antibiotic resistance.

**Methods**

**Bacterial strains and growth conditions.** Unless stated otherwise, all chemicals and reagents were purchased from Sigma Aldrich. M. tuberculosis H37Rv, 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. Appropriate kanamycin or hygromycin was added at 25 or 50 μg mL⁻¹, respectively. Mycobacterium smegmatis strains mc²155 and mc²155 ΔnamB were maintained on Tryptic Soy Broth or Tryptic Soy Agar where appropriate.40 For growth on defined carbon sources, strains were cultivated in Sauton’s minimal medium containing per 1 L, 4 g asparagine, 2 g citric acid, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, and 0.05 g ferric ammonium citrate, 0.05% tyloxapol and 5 mM of each carbon source unless stated otherwise.42 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. GlcNAc-MurNac was purchased from Toronto Research Chemicals.

**Cloning and purification of LpqITB.** The gene encoding LpqI (Rv0237) was amplified from M. tuberculosis H37Rv genomic DNA using standard PCR conditions with the lpqITBSUMOF and lpqITBSUMOR primers and cloned into the T-A site of the Champion pET-SUMO expression plasmid (Invitrogen) according to the manufacturer’s specifications (all primers are found in Supplementary Table 1). For production of LpqI 1 L of E. coli BL21 [pLPqITB] grown in Terrific Broth to an OD₆₀₀ 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 Tris·HCl, 300 mM NaCl, 10 mM imidazole pH 7.8 and lyzed 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 Tris·HCl, 300 mM NaCl, 10 mM imidazole pH 7.8 in the presence of recombinant Ulp1 protease, which specifically cleaves the His-SUMO tag. Digested protein was passed through a second IMAC column (1 mL HisTrap FF, GE Healthcare) and the flow-through fraction was found to contain pure, untagged LpqI. Purified protein was dialysed into 25 mM Bis-Tris pH 6.5, 100 mM NaCl.

**Cloning and purification of RpfB.** RpfB was cloned according to the same procedure as LpqI using the primers RpfBFwd and RpfBRev. Expression and purification was achieved using the same general procedure as LpqI.

**Crystallography.** Prior to crystallisation, LpqI was concentrated to 20 mg mL⁻¹ in 25 mM Bis-Tris pH 7.5, 100 mM NaCl. LpqI crystals were grown by the sitting-drop vapour diffusion method by mixing an equal volume of protein solution with re-aeration after re-activation from non-replicating persistence in the Wayne hypoxia model.41

The fate of GlcNAc in this pathway remains unclear, although our data and prior observations suggest that the bacteria do not re-use this sugar. The inability to utilise this sugar is surprising given the conservation of the nagA (Rv3332) gene in mycobacteria.42 However, it is possible that an alternative GlcNAc-recovery pathway exists that was not functional under the conditions we have tested. This is hinted at, with our ¹⁴C-labelled muropeptides, where incorporation of the labelled-GlcNAc is not expected, given the lack of monomeric GlcNAc utilisation by the cells. During the production of radio-labelled PG in M. smegmatis, a portion of the GlcNAc will have been used by the bacterium to generate MurNac rather than strict shunting of GlcNAc into UDP-GlcNAc for cell wall biosynthesis. The subsequent removal of the lactyl-ether from this ¹⁴C-MurNac by M. bovis BCG would then follow steps and intermediates we do not yet fully understand. Indeed, bacterial etherases comprise a diverse number of mechanisms and potential reaction products and a product other than free GlcNAc is entirely possible.43 We are currently trying to identify and characterise the enzyme responsible for the observed lactyl-etherase activity.

Fig. 6 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. Some of this material undergoes limited release to stimulate the host immune system. The remainder are subsequently degraded by amidases and other peptidases. LpqI then cleaves GlcNAc-MurNac, which is followed by lactyl-ether cleavage. Lactate can then be used by the cell under aerobic conditions and GlcNAc (or its derivatives) are most likely released. Perturbation of this system by deleting LpqI leads to increased resistance to anti-mycobacterial agents.
I.1 m sodium malonate, 0.1 m HEPES, 0.5% w/v Jaffeline ED-2001 (pH 7.0). Crystals were cryo-protected with a saturated solution of sodium malonate and flash-cooled in liquid nitrogen. X-ray data were collected at beamline 104-1 of the Diamond Light Source, Oxford. Data were auto-processed using Xia2 and general file manipulations were performed using the CCP4 suite of programmes45,46. The structure was phased by molecular replacement using the unpublished M. smegmatis LpqI structure (PDB: 4YFV, 67% sequence identity) using the programme Phaser57,58 (version 1.7). The structure was subsequently built in PHENIX and the remaining parts were built in COOT with further refinement using PHENIX and PDB-REDO49–51.

Kinetic characterisation of LpqI. Purified LpqI was evaluated for glycolide hydrolase activity using a variety of substrates. As an initial screening assay, LpqI was incubated at 1 μM with either 4-methylumbelliferyl (4MU) or p-nitrophenoxy (pNP) derivatives of a variety of sugars (arabinose, galactose, galactosamine, arabino-nafurorace, rafucose, mannose, N,N′-diaminocitrate, N-acetyl-ylglucosamine) in Bis-Tris pH 7.5, 100 mM NaCl at 37 °C. The release of pNP was followed by change in absorbance at 420 nm, whereas production of 4-MUP was monitored using the fluorescence method (Ex. 355 nm; Em. 460 nm) in a BMG FLUOstar plate reader. Kinetic characterisation of LpqI was conducted using varying concentrations of 4MU-GlcNac. The raw data were compared to standards of pNP or 4MU. All data were analysed using GraphPad Prism 7.

Substrate specificity of LpqI. To determine whether LpqI could act as an autolysin and cleave insoluble mycobacterial PG we labelled insoluble PG from M. smegmatis mc²155 with fluorescein isothiocyanate50. LpqI, mutanolysin or a chitinase were incubated at a final concentration of 1 μM overnight with 1 μg mL⁻¹ FITC-PG conjugate in 25 mM sodium phosphate buffer pH 6.5. The sample was then filtered and the fluorescence of the soluble fraction was read (Ex. 495, Em. 519) in a BMG Fluostar plate reader.

Digestion of wall material with mutanolysin or RpfB was carried out overnight at 37 °C in 20 mM ammonium acetate buffer (pH 6.5) with continuous mixing. Following digestion, solubilised muropeptides were isolated using graphitised carbon solid-phase-extraction cartridges51. Purified fractions were evaporated to dryness and the concentration of reducing sugars in the pool of soluble muropeptides was assessed using the 3,methy1-benzothiazolinone hydrazone assay52. For RpfB-generated muropeptides the concentration was estimated gravimetrically using an average molecular weight of 953.45 Da for GlcNac-1,6-anhydroMurNglc-tetrapeptide.

Reactions including 1 μM LpqI, and 0.5 mM mutanolysin-derived PG fragments or 0.1 mM RpfB-derived PG fragments in 25 mM ammonium acetate buffer pH 6.5 were incubated for 18 h at 37 °C. The reactions were then evaluated using a 2-step assay. Instead of the 2- or 3-step protocols published for the synthesis of 4MU-o-lactate previously, we used a simplified one step method53,54. In total, 1.5 g of (s)-bromopropionic acid was added to 1 g of 4-methylumbelliferyl ester (MUC) in 40 mL anhydrous dimethylformamide and 0.75 g CaCO₃. This was stirred at room temperature overnight and the product was extracted for three times with water/ethyl-acetate and the organic phase was dried over sodium sulphate. 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.

Turnover of 4MU reporter compounds by M. bovis BCG. To test turnover of 4MU-GlcNac or 4MU-o-lactate by whole cells, 100 μL of a mid-exponential culture (OD₆₀₀ = ~0.6) was added to a sterile 96-well plate in Sauton’s minimal media supplemented with 0.5% Tween and 1% glycerol in addition to 1 mM 4MU-o-lactate or 4MU-GlcNac. Similar controls lacking cells or the reporter compound were included as well. This was incubated at 37 °C and mixed at 300 rpm. On the indicated days the 4MU fluorescence of the sample was read on a BMG PolarStar microplate reader with a constant gain setting.

Mutant generation. To generate the ΔlpqI strain we used specialised transduction according to established protocols47. A recombinant lpqI knockout phage was designed to replace the chromosomal lpqI gene using homologous flanking regions to lpqI with a sacB gene and a hygromycin resistance cassette in-between using the LL-φpIβPCG, LR-φpIβPCG, RR-φpIβPCG, and RL-φpIβPCG primers. The resulting phage was transduced into M. bovis BCG and transductants were selected on 7H11 agar plates containing 75 μg mL⁻¹ hygromycin. The mutant was verified by PCR and phenotypically with 4MU-GlcNac where loss of lpqI was expected to abolish turnover of this fluorescent substrate. The complemented strain was generated by incorporation of the lpqI-Δ′ and 150bp 5’ to the start codon containing the promoter sequence into the promoter-less integrative mycobacterial shuttle vector pMV306 using primers φpIβPCGCompF and φpIβPCGCompR to generate the resulting pMV306-ΔpI plasmid53. This was electroporated into M. bovis BCG ΔlpqI and transformants were selected on 7H11 agar containing hygromycin and kanamycin. The complemented mutant was verified phenotypically with 4MU-GlcNac. A control strain was also generated using the empty pMV306 vector with the same protocols.

Antimicrobial testing. Mid-exponential cultures of M. bovis BCG and derivative strains were diluted to OD₆₀₀ = 0.1 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% v/v resazurin and 12.5 μL of 20% Tween 80 v/v was added to the culture. This was incubated overnight 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 mycobacterial cell wall. Rapid purification of cell wall from small cultures was carried out using a modified phenol extraction protocol19. 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 Perccell Evolution Bead Beater at 2000 rpm for 3 min. The lysate was then transferred to glass culture tubes to which 2 mL of 98% phenol was added and vortexed for 1 min. This was heated for 1 h at 70 °C, allowed to cool and the insoluble material was collected by centrifugation at 3220 × g. The aqueous phase was removed and 4 mL of methanol was added. This was vortexed and centrifuged again. Finally, the pellet was washed three times with methanol and once with water before being freeze or used for subsequent enzymatic treatment.

Large-scale purification of PG. Purification of PG from M. smegmatis was achieved following established protocols54. Six liters of M. smegmatis were grown with aeration to mid-exponential phase (OD₆₀₀ = 0.6) 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% sodium dodecyl sulphate (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-polyglycolic acid (mAG). The mAG was incubated for 4 days in 0.5% (w/v) KOH in methanol at 37 °C before being washed through three times with 5% (v/v) acetic acid. The mycolic acids were extracted with three washes of diethyl ether. The phosphodiester linker the AG-PG complex was cleaved using 0.2 mM H₂SO₄ and the PG was separated from the solubilised AG by centrifugation prior to neutralisation with NaCO₃ and washed with water three times. The insoluble PG pellet was sequentially digested with a-amylase (100 μg mL⁻¹), DNase (10 μg mL⁻¹) and RNase (5 μg mL⁻¹) for 8 h before protease 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 seven times). The resulting material was lyophilised and stored at −20 °C until it was needed. Digestion and quantification of this material was carried out as described above.

Synthesis of 4MU-o-lactate. Instead of the 2- or 3-step protocols published for the synthesis of 4MU-o-lactate previously, we used a simplified one step method55,56. In total, 1.5 g of (s)-bromopropionic acid was added to 1 g of 4-methylumbelliferyl ester (MUC) in 40 mL anhydrous dimethylformamide and 0.75 g CaCO₃. This was stirred at room temperature overnight and the product was extracted for three times with water/ethanol-acetate and the organic phase was dried over sodium sulphate. 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.

Data availability. The atomic coordinates and structure factors of LpqI are available from Protein Data Bank with accession code 6GFV. All data used to generate the figures are available in the Source Data file. Other data that support the findings of this study are available from the authors on request.
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Author contributions
Conceived of the study: P.J.M. conceived and designed the experiments: P.J.M., G.V.M., A.L.L., G.S.B. performed the experiments: P.J.M., I.T.C, N.V., M.J., M.C. analysed the data: P.J.M., I.T.C, N.V., M.C, G.V.M., A.L.L., G.S.B. wrote the manuscript: P.J.M. edited the manuscript: P.J.M, G.V.M., A.L.L., G.S.B.

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