Comparative genomics for mycobacterial peptidoglycan remodelling enzymes reveals extensive genetic multiplicity

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Abstract

Background: Mycobacteria comprise diverse species including non-pathogenic, environmental organisms, animal disease agents and human pathogens, notably Mycobacterium tuberculosis. Considering that the mycobacterial cell wall constitutes a significant barrier to drug penetration, the aim of this study was to conduct a comparative genomics analysis of the repertoire of enzymes involved in peptidoglycan (PG) remodelling to determine the potential of exploiting this area of bacterial metabolism for the discovery of new drug targets.

Results: We conducted an in silico analysis of 19 mycobacterial species/clinical strains for the presence of genes encoding resuscitation promoting factors (Rpfs), penicillin binding proteins, endopeptidases, L,D-transpeptidases and N-acetylmuramoyl-L-alanine amidases. Our analysis reveals extensive genetic multiplicity, allowing for classification of mycobacterial species into three main categories, primarily based on their rpf gene complement. These include the M. tuberculosis Complex (MTBC), other pathogenic mycobacteria and environmental species. The complement of these genes within the MTBC and other mycobacterial pathogens is highly conserved. In contrast, environmental strains display significant genetic expansion in most of these gene families. Mycobacterium leprae retains more than one functional gene from each enzyme family, underscoring the importance of genetic multiplicity for PG remodelling. Notably, the highest degree of conservation is observed for N-acetylmuramoyl-L-alanine amidases suggesting that these enzymes are essential for growth and survival.

Conclusion: PG remodelling enzymes in a range of mycobacterial species are associated with extensive genetic multiplicity, suggesting functional diversification within these families of enzymes to allow organisms to adapt.

Keywords: Peptidoglycan, Transglycosylases, Amidases, D,D-carboxypeptidases, Transpeptidases, Endopeptidases

Background

Bacteria inhabit every environment on earth with a resilience that is central to their survival and consequently, they continue to serve as a major source of human disease. A critical factor, which has been central to the success of these organisms, is the diversity entrenched within their cell walls, which serves as a major barrier to drug treatment. The mycobacterial cell wall is an incredibly complex structure, with multiple layers that collectively constitute a waxy, durable coat around the cell, which serves as the major permeability barrier to drug action [1-4]. Considering this, the cell wall and related components are attractive for the mining of new drug targets, and remain relatively unexploited for drug discovery in the case of certain bacterial pathogens [2,5,6]. Peptidoglycan (PG or the murein sacculus) is a rigid layer that constricts the cell membrane and the cell within, providing mechanical stability to counteract imbalances of cytoplasmic turgour pressure, and plays an important role in determining cell size and shape [7-10]. Mycobacteria possess a highly complex additional lipid rich outer membrane, with different constituents anchored either directly to the cell membrane or to the PG [6,11]. Arabinogalactan (AG), a structure unique to actinomycetes, is bound externally to an N-acetyl muramic acid (NAM) moiety of the PG [3,12]. In mycobacteria, a certain proportion of the muramic acid is N-glycolylated [13] through the activity of NamH, a UDP-N-acetylmuramic
acid hydroxylase [14]. This modification results in altered tumour necrosis factor α production [15,16] however, abrogation of NamH activity does not lead to decreased virulence in mice [16].

This serves as an anchor for further lipid rich cell wall components, either by covalent attachment to the mycolic acid layer or through non-covalent interactions [trehalose dimycolate (TDM); phthiocerol dimycocerosate (PDIM); phenolic glycolipids (PGL)] [3,11,12]. PG consists of repeated alternating sugars N-acetyl glucosamine (NAG) and NA/GM (muramic acid with or without the glycolyl modification), which are linked to a pentapeptide side chain [7-9,17], Figure 1. The crosslinking of these subunits lead to a lattice-like structure around the cell.

The PG in bacterial cell walls is an incredibly dynamic structure that requires constant expansion and remodelling during growth to accommodate the insertion of new PG subunits, secretion apparatus, flagellae etc. [9,10]. During cell division, pre-septal PG synthesis and subsequent degradation of the septum is critical to daughter cell separation; consequently these processes are carefully regulated [7]. In this regard, there is a diversity of enzymes involved in cross-linking, degradation and remodelling of PG, which are illustrated in Figure 1. A ubiquitous feature in bacteria is the genetic multiplicity associated with these functions, which presumably contributes to the ability of different organisms to adapt under varying environmental conditions [7,9,10]. In the case of Mycobacterium tuberculosis, the causative agent of tuberculosis, there is a dire need for new drugs with novel modes of action [7]. The increased prevalence of drug resistant strains has raised concerns regarding the sustainability of the current treatment regimen. To address this, several aspects of mycobacterial metabolism are being assessed for potential new drug targets [18]. The genetic redundancy associated with PG biosynthesis together with the reliance on robust bacterial growth to achieve significant drug target vulnerability, has hampered drug development initiatives that target the cell wall [19]. For other bacterial pathogens, PG has been successfully used as an antibiotic target in the past, as evidenced by the widespread use of β-lactam antibiotics among others, the biosynthesis and degradation of this macromolecule in mycobacteria is meritorious of further investigation.

In this study, we undertake a comprehensive analysis of the genomic repertoire of PG remodelling enzymes in various pathogenic and environmental mycobacteria to determine the level of genetic multiplicity/redundancy and degree of conservation. We focus on those enzymes involved in cross-linking and remodelling of the PG in the periplasmic compartment, including: resuscitation promoting factors (RpfSs), penicillin binding proteins (PBPs), transpeptidases, endopeptidases, and N-acetylmuramoyl-

L-alanine amidases. Our data reveal extensive genetic multiplicity for the 19 strains analysed in this study, which allowed grouping of strains into three families based on their complement of PG remodelling enzymes, including the MTBC, other pathogenic mycobacteria and non-pathogenic environmental organisms.

Results and Discussion

The comparative genomics analysis for PG remodelling enzymes in mycobacterial species obtained from this study is summarised in Table 1. We analysed 19 distinct species/strains: Six of these belong to the MTBC, six are classified as other pathogenic bacteria [three of which belong to the Mycobacterium avium complex (MAC)] and six environmental species including Mycobacterium smegmatis. Mycobacterium leprae is listed separately due to its substantially reduced genome which emerges as an outlier in the analysis.

Resuscitation promoting factors (lytic transglycosylases)

Of all the enzymes identified in this study, the Rpf family is the most extensively studied. This group of enzymes are of particular interest due to demonstrated importance for reactivation from dormancy and essentiality for growth in Micrococcus luteus [22,23]. Whilst Mi. luteus encodes a single, essential rpf gene, mycobacteria encode a multiplicity of rpf homologues and those present in M. tuberculosis, designated as rpfA-rpfE, encode closely related proteins all of which retain the Rpf domain [24-26], Figure 2. These have been the subject of intense study due to the potential role they may play in reactivation disease in individuals that harbour latent TB infection [25-27,31]. In this regard, the five rpf genes present in M. tuberculosis are collectively dispensable for growth but are differentially required for reactivation from an in vitro model of non-culturability [32,33]. Furthermore, the RpfSs are combinatorially required to establish TB infection and for reactivation from chronic infection in mice [32-35]. For additional information, the reader is referred to several extensive reviews on this topic [25,27,28,36-38].

RpfSs are classified as lytic transglycosylases (LTs) based on sequence conservation and three-dimensional protein structure [29,39-41]. LTs cleave the β-1,4-glycosidic bonds between the NAG-NA/GM sugar subunits, Figure 1, and their activity is required for insertion of new PG units and expansion of the glycan backbone [9]. In mycobacteria RpfB contains a lysozyme-like, transglycosylase-like PFAM domain, and consequently this group of enzymes are predicted to cleave the glycan backbone of PG [39-41]. Direct evidence for this is lacking and moreover, the mechanism through which Rpf-mediated cleavage of PG results in growth stimulation remains unknown. The repertoire of rpf genes is highly conserved in the MTBC; in contrast,
other pathogenic mycobacteria lack rpfD, including M. leprae, Table 1. Based on the distribution of rpfC and rpfD, we categorize the 19 strains analysed in this study into the MTBC (which retains all five rpf homologues present in M. tuberculosis), other pathogenic mycobacteria (which lack rpfD) and environmental strains (which lack both rpfC and rpfD). This classification is supported by phylogenetics analysis which confirms these clusters and duplication/loss of genes, Additional file 1: Figure S1. Recently, it has been shown that the RpfS can serve as potent antigens [42] and Rpf-directed host immune responses allow for detection of TB in latently infected individuals [43]. It is noteworthy that strains lacking different combinations of rpf genes confer significant protective efficacy when used as vaccine strains in mice [44]. Hence, any variation in rpf gene complement between pathogenic mycobacteria may have significant consequences for broadly protective effects of future Rpf-based vaccines.

The environmental species retain three rpf genes [rpfA, rpfB (duplicated in Mycobacterium sp. JLS, Mycobacterium

![Figure 1](http://www.biomedcentral.com/1471-2180/14/75)
# Table 1 Genetic complement for PG remodelling enzymes in 19 mycobacterial species

| MTB complex | Other mycobacterial pathogens | Environmental mycobacterial species |
|-------------|-----------------------------|-------------------------------------|
| M. tuberculosis H37Rv | M. avium 104 | M. smegmatis mc²155 |
| M. bovis BCG Pasteur 1173P | M. avium sp. subspec. K10 | M. vanbaalenii PRy-1 |
| | OCM 13500 | M. sp. MCS |
| | M. ulcerans AG9/99 | M. sp. KMS |
| | M. marinum | M. sp. JLS |
| | | M. gihum PYR-GCK |
| | | M. leprae TN |

| Resuspension promoting factors | Penicillin binding proteins | Endo-Peptidases |
|-------------------------------|---------------------------|----------------|
| ppaA | Rv0050 Nva_0085 | MMEG_0100 |
| ppaB | Rv0069 MRA_0058 | MMEG_0101 |
| ppaC | Rv0079 MRA_0059 | MMEG_0102 |
| ppaD | Rv0080 MRA_0060 | MMEG_0103 |
| ppaE | Rv0081 MRA_0061 | MMEG_0104 |
| ppaF | Rv0082 MRA_0062 | MMEG_0105 |
| ppaG | Rv0083 MRA_0063 | MMEG_0106 |
| ppaH | Rv0084 MRA_0064 | MMEG_0107 |
| ppaI | Rv0085 MRA_0065 | MMEG_0108 |
| ppaJ | Rv0086 MRA_0066 | MMEG_0109 |
| ppaK | Rv0087 MRA_0067 | MMEG_0110 |
| ppaL | Rv0088 MRA_0068 | MMEG_0111 |
| ppaM | Rv0089 MRA_0069 | MMEG_0112 |
| ppaN | Rv0090 MRA_0070 | MMEG_0113 |
| ppaO | Rv0091 MRA_0071 | MMEG_0114 |
| ppaP | Rv0092 MRA_0072 | MMEG_0115 |
| ppaQ | Rv0093 MRA_0073 | MMEG_0116 |
| ppaR | Rv0094 MRA_0074 | MMEG_0117 |
| ppaS | Rv0095 MRA_0075 | MMEG_0118 |
| ppaT | Rv0096 MRA_0076 | MMEG_0119 |
| ppaU | Rv0097 MRA_0077 | MMEG_0120 |
| ppaV | Rv0098 MRA_0078 | MMEG_0121 |
| ppaW | Rv0099 MRA_0079 | MMEG_0122 |
| ppaX | Rv0100 MRA_0080 | MMEG_0123 |
| ppaY | Rv0101 MRA_0081 | MMEG_0124 |
| ppaZ | Rv0102 MRA_0082 | MMEG_0125 |

**Note:** The table continues with detailed entries for each gene and its corresponding function, sequence, and other relevant information.
Table 1 Genetic complement for PG remodelling enzymes in 19 mycobacterial species (Continued)

| ldtMt | Rv0192 | MRA_0200 | MT3202 | MAF_01910 | Mb0198 | BCG_0229 | MAV_4986 | MAP3634 | OCLL_48990 | MUL_1085 | MMAR_0435 | MBA_4537c | MSMEG_0233 | Mvan_3694 | Mvan_0177 | Mmcs_0151 | Mkms_0160 | Mjls_4535 | Mjls_0141 | Mflv_5330 | Mflv_0344 |
|-------|--------|----------|--------|------------|--------|----------|----------|--------|-------------|---------|-----------|----------|------------|----------|----------|----------|--------|----------|----------|----------|----------|----------|----------|
|       |        |          |        |            |        |          |          |        |             |         |           |          |            |          |          |          |        |          |          |          |          |          |
| ami1  | Rv0873 | MRA_0400 | MT3501 | MAF_04870 | Mb0493 | BCG_0524 | MAV_4666 | MAP3976 | OCLL_45320 | MUL_4553 | MMAR_0809 | MBA_4061c | MSMEG_0929 | Mvan_0824 | Mmcs_0654 | Mmcs_0967 | Mjls_0047 | Mjls_0208 | ML2446   |
| ami2  | Rv0915 | MRA_3954 | MT4034 | MAF_39300 | Mb3946 | BCG_0021 | MAV_5303 | MAP4341 | OCLL_51370 | MUL_5068 | MMAR_5479 | MBA_6935c | MSMEG_5404 | Mvan_5493 | Mjls_5780 | Mjls_0857 | ML2704   |
| ami3  | Rv3811 | MRA_3851 | MT3918 | MAF_38260 | Mb3841 | BCG_3873 | MAV_2026 | MAPD209c | OCLL_21600 | MUL_4995 | MMAR_5575 | MBA_6406c | MSMEG_5510 | Mvan_5510 | Mmcs_5022 | Mmcs_5110 | Mjls_5403 | Mjls_1157 |
| ami4  | Rv3594 | MRA_3633 | MT3700 | MAF_36070 | Mb3625 | BCG_3659 | -        | -      | -            | -       | -         | -        | -          | -        | -        | -        | -      | -        | -        |

The names of the various organisms analysed are shown in the columns and gene complement is given in the corresponding rows. Mycobacteria are grouped as M. tuberculosis Cluster (MTBC), other pathogens, environmental species and M. leprae. Genes are sorted by functional groups in rows. The listing of a gene is based on its presence by protein BLAST analysis, either at curated sites or directly at NCBI. For all genes the protein sequence, in FASTA format, was obtained and utilised for phylogeny. Annotations for M. africanum (MAF_) and M. intracellulare (OCU_) were obtained directly from NCBI. BLAST analysis was performed against individual strains at NCBI using M. tuberculosis H37Rv homologues as the query sequence. The cut off was taken at a coverage of >90% and an identity of >40%. MSMEG_1900 was identified at SmegmaList. In the case of ripD, parentheses indicate the 63C-terminal amino acid truncation. Further in-depth information, and confirmation of gene annotation, was obtained by assessment of phylogeny based on protein sequences, Additional file 1: Figure S1-S7. Font differences in the M. tuberculosis H37Rv column indicate genes that have been annotated as essential by two different TraSH analyses – indicated in bold (Sassetti et al. [20]) and/or italicised (Griffin et al. [21]) are those genes identified as essential or required for optimal growth.
sp. KMS, Mycobacterium sp. MCS) and rpfE, Table 1 and Additional file 1: Figure S1. Although rpfC (Rv1884c in M. tuberculosis) homologues have been annotated as present in all mycobacteria [45], our analysis shows that the M. tuberculosis rpfC homologue is absent from environmental species. Artemis Comparison Tool (ACT) whole genome alignment reveals that the region encoding rpfC in M. tuberculosis is absent in M. smegmatis and all other environmental bacteria (data not shown). Thus, based on gene synteny, there is no direct rpfC homologue in these strains. However, there is a local duplication of rpfE in all the environmental strains (annotated as MSMEG_4643 in M. smegmatis), Table 1, Additional file 1: Figure S1. Consequently, we re-annotate MSMEG_4640 to rpfE2, as a homologue of MSMEG_4643, rather than a homologue of Rv1884c. As RpfE interacts with the Rpf Interacting Protein A (RipA) [46], there may be some functional consequence to the presence of multiple copies in M. smegmatis and other environmental bacteria.

The restriction of rpfC and rpfD homologues to pathogenic and MTBC strains, along with the duplication of rpfB in some environmental species, raises interesting questions regarding the nature of growth stimulation in these organisms. These differences suggest that the latter require fewer secreted Rpfs and are more reliant on the membrane bound RpfB homologue. This could be related to the fact that environmental organisms are required to grow in diverse niches of varying size and complexity making them more dependent on localised growth stimulatory activity through a membrane bound
Rpf rather than paracrine signalling from diffusible RpfS produced by neighbouring organisms. It is noteworthy that of all five homologues in *M. tuberculosis*, deletion of *rpfB* individually or in combination with *rpfA* results in colony forming defects and prolonged time to reactivation from chronic infection in mice [21,34,35].

The role of RpfS in TB disease in humans remains enigmatic. It has been demonstrated that sputum from patients with active TB disease, before the initiation of treatment, is characterised by a population of dormant bacteria that require RpfS for growth [47]. These data provide tantalizing preliminary evidence that RpfS play an important role in determining bacterial population dynamics in TB infected patients and moreover are critical for disease transmission. Within the granulomatous environment, it may be preferable for the bacterial population as a whole to facilitate emergence of fitter clones which are able to exit from arrested growth. This could explain clonal emergence in clinical samples if few strains are able to expand sufficiently to cause tubercular lung disease.

**Penicillin binding proteins**

Penicillin Binding proteins (PBPs) are a large family of evolutionarily related cell wall associated enzymes, that bind β-lactam antibiotics [48,49]. PBPs are classified according to their molecular weight as either high molecular mass (HMM) or low molecular mass (LMM) and are studied herein proved difficult. Consequently, we have not analysed these genes further.

In mycobacteria, Class A PBPs constitute bi-functional enzymes designated as ponA1 (PBP1, Rv0050, [50]); and ponA2 (PBP1A, Rv3682 [51]), Table 1. They contain separate domains for transpeptidase and transglycosylase activities. Both these genes are present in all mycobacteria and, as previously reported for *M. smegmatis* and other environmental strains, there is a duplication of ponA2 which was annotated as ponA3 [51], Table 1 and Additional file 1: Figure S2.

Class B PBPs proteins PbpA (pbbA; Rv0016c, [52]), PbpB (pbbB; Rv2163c, [53]) and PBP-lipo (Rv2864c, [49]) are predicted to contain only transpeptidase domains and possibly additional dimerisation domains, but lack transglycosylase activities, Figure 2. Both PbpA and PbpB (FtsI) are involved in progression to cell division in *M. smegmatis* where gene deletion or depletion manifests in altered cell morphology and antibiotic resistance profiles [52]. In this family of PBPs – as exemplified by ponA2 - there is a distal duplication of PBP-lipo in the environmental strains, Table 1 and Additional file 1: Figure S3. No experimental data on this are currently available, but the lipophilic domain is speculated to allow for cell wall association.

**Endopeptidases**

Endopeptidases are enzymes that cleave within the stem peptides in PG. In this study, we focus on the Nlp/P60 class of endopeptidases, which cleave within the stem peptides between positions 2 and 3 as exemplified by RipA, Figure 1. RipA is an essential PG hydrolytic enzyme that synergistically interacts with RpfB and RpfE [46,57] to form a complex that is able to degrade PG. The RipA-RpfB hydrolytic complex is negativelyregulated by PnaA2 [58] suggesting a dynamic interplay between PG hydrolases, one that would be significantly nuanced with the presence of multiple RipA and Rpf homologues. In this regard, our analysis reveals four endopeptidases in *M. tuberculosis* that display strong homology to ripA, Table 1, Figure 2, Additional file 1: Figure S5. With the exception of *Mycobacterium abscessus* and *M. leprae*, pathogenic mycobacteria retain all five of these homologues. Environmental strains display enhanced expansion of endopeptidases, with the exception of the ripD homologue (Rv1566c). The functional consequence of this remains unknown but it is noteworthy that these strains have also expanded their *rpfE* and *rpfB* gene repertoire, suggesting that the multiplicity in this
case allows for a greater number of RipA-RpfB/E protein complexes, as well as for protein complexes with different subunit composition. Dysregulated expression of RipA leads to dramatic alterations in cellular morphology and growth [59] suggesting that careful regulation of this protein, both at the expression level as well as by post-translational level is essential. Genetic expansion of RipA homologues along with two copies of RpfB and RpfE, both of which interact with RipA implies a functional consequence of this expansion. In addition, strong regulation of these multiple copies would be required to prevent any detrimental effects on cell growth.

RipB displays strong sequence homology RipA in M. tuberculosis (100% amino acid identity over 58% coverage) and similar domain organization [60], but lacks the N-terminal motif, Figure 2, that has been implicated in auto inhibition by blocking the active site in the three-dimensional crystal structure [61]. More recently, high resolution crystal structures of RipB and the C-terminal module of RipA (designated as RipAc) revealed striking differences in the structure of these proteins, specifically in the N-terminal fragments that cross the active site [60]. Both RipB and RipAc are able to bind high molecular weight PG and retain the ability to cleave PG with variable substrate specificity, which is not regulated by the presence of the N-terminal domain [60]. This suggests that the N-terminus does not regulate PG degrading activity and in this context, the physiological consequences of the reduced size of RipB and RipD, Figure 2, remain unknown. The high degree of conservation of RipB across all pathogenic mycobacteria including M. leprae, Table 1, Additional file 1: Figure S5 indicates that variable substrate specificity in PG hydrolases in essential for pathogenesis. The Mycobacterium marinum homologues of Rv1477 and Rv1478, iipA and iipB (MMAR_2284 and MMAR_2285 respectively), Table 1, Additional file 1: Figure S5, have been implicated in macrophage invasion, antibiotic susceptibility and cell division [62]. As with the other enzymes assessed in this study, environmental mycobacteria display greater genetic multiplicity for these homologues, Table 1.

Structural analysis of RipD reveals alterations in the catalytic domain, consistent with the inability of this protein to hydrolyse PG [63]. Nevertheless the core domain of RipD is able to bind mycobacterial PG and this binding is negatively regulated by the C-terminal region [63]. However, RipD homologues in the environmental mycobacteria lack the 63C-terminal amino acids, Table 1 (shown in parentheses), possibly allowing for stronger binding of this enzyme to PG.

Rv2190c encodes another NlpC/P60-type PG hydrolase in mycobacteria. Deletion of this gene in M. tuberculosis results in altered colony morphology, attenuated growth in vitro, defective PDIM production and reduced colonisation of mouse lungs in the murine model of TB infection [64]. Consistent with this, homologues of Rv2190c are found in all pathogenic mycobacteria, Table 1, with notable genetic expansion in some environmental species. In contrast, the Rv0024 is absent from environmental species, suggesting that it could be required for intracellular growth or some other component of the pathogenic process, Table 1, Additional file 1: Figure S5.

LD - Transpeptidases

L,D-transpeptidases (Ldt) are a group of carbapenem sensitive enzymes in M. tuberculosis [56] that contribute to the formation of a 3 → 3 link between the two adjacent mDAP (mDap → mDap bridges) residues in PG, distinct from the classic 4 → 3 link (D-Ala → mDAP), Figure 1. M. abscessus [65] and M. tuberculosis [66] exhibit increased ratios of the 3 → 3 cross-link in stationary axenic culture, indicating that mycobacteria are capable of modulating their PG at the level of transpeptidase in response to growth stage and the availability of nutrients. Both Ldt Mt1 and Ldt Mt2 (Rv0116c and Rv2518c respectively) were experimentally shown to affect M. tuberculosis H37Rv morphology, growth characteristics and antibiotic susceptibility in vivo [67]. The crystal structure of Ldt Mt2 places the extramembrane domain 80–100 Å from the membrane surface and indicates that this enzyme is able to remodel PG within this spatial region of the PG sacculus [68]. More recently, it has been demonstrated that the combinatorial loss of both Ldt Mt1 and Ldt Mt2 in M. tuberculosis resulted in morphological defects and altered virulence in the murine model of TB infection [69]. A notable variability of L,D-transpeptidase genes is found in mycobacteria, Table 1, Figure 2 and Additional file 1: Figure S6. Five homologues are present in all but one pathogenic strain, while multiple homologues are evident in most environmental strains. The exception is Ldt M3 (Rv1433), which is absent from the pathogen Mycobacterium ulcerans and from the environmental species Mycobacterium vanbaalenii, M sp. MCS and M. sp. KMS, yet its presence in M. leprae suggests functional importance. As with RipA, M. gilvum shows the greatest expansion of the ldt genes. Biochemical characterisation of all five M. tuberculosis H37Rv homologues, Ldt Mt1 - Ldt Mt5 confirms PG cross-linking and/or β-lactam acylating enzyme activities in all of these enzymes [70]. This activity can be abolished by treatment with imipenem and cephalosporins, indicating that this group of enzymes holds great promise for TB drug development [70,71]. Moreover, the functionality of all the Ldt homologues present in M. tuberculosis raises interesting questions with respect to the functional consequences of the expansion of this protein family in environmental strains, which may require greater flexibility in Ldt function.
Amidases
While endopeptidases and transpeptidases are responsible for cleavage within or between peptide stems, amidases act to remove the entire peptide stem from the glycan strands, cleaving between the NA/GM moiety and the L-Ala in the first position of the stem peptide, Figure 1. The amidases have been implicated in PG degradation, antibiotic resistance/tolerance and cell separation in *Escherichia coli* and other organisms, and can be organized into 2 main families containing either an amidase_2 or amidase_3 – type domain [8,9,72]. The amidases of *E. coli* (which retains 5 amidases designated AmiA, AmiB, AmiC, AmiD and AmpD) have specific substrate requirements governed by the structural confirmation of the NAM carbohydrate moiety. Knockout of these amidases results in chaining phenotypes, abnormal cell morphologies and/or increased susceptibility to certain antibiotics [72-74]. Amidases have also been implicated in spore formation, germination and cell communication in *Bacillus subtilis* [75,76]. The role of amidases in mycobacterial growth, virulence and resuscitation from dormancy is unknown and any impact of these on mycobacterial morphology and antibiotic resistance remains to be demonstrated. Analysis of the amidase gene complement in mycobacteria reveals the presence of four homologues in *M. tuberculosis*, two containing the amidase_2 domain (ami3; Rv3811 and ami4; Rv3594) and two the amidase_3 domain (ami1; Rv3717 and ami2; Rv3915), Table 1, Figure 2 and Additional file 1: Figure S7. The crystal structure of Rv3717 from *M. tuberculosis* confirms that this enzyme is able to bind and cleave muramyl dipeptide [77]. The amidase family distinguishes itself from all other enzyme families by absence of a homologue (ami4) from non-MTBC pathogens and its presence in the MTBC and environmental strains. *M. leprae* retains only the ami1 and ami2 genes – both containing the amidase_3 domain. This suggests that amidase_2 domain amidase activity is dispensable specifically in this species, but required for peptidoglycan remodelling in the other pathogenic mycobacteria.

*Mycobacterium leprae*
Very little is known about *in vitro* growth and division of *M. leprae*, as it can only be grown in animal models. From our analysis, it is apparent that *M. leprae* harbours notable genetic redundancy for PG remodelling enzymes (Table 1) in contrast to its minimal gene set for other areas of metabolism [78]. Considering that PG subunits or precursors cannot be scavenged from the host, it is expected that pathogenic bacteria would retain complete pathways for biosynthesis and remodelling of PG. However, the presence in *M. leprae* of multiple homologues within each class of PG remodelling enzyme assessed in this study, suggests that some level of multiplicity is required to ensure substrate flexibility. Further work in this regard is difficult due to the limited tractability of *M. leprae* for *in vitro* manipulation.

Conclusions
Mycobacteria represent a wide range of species with a great variety of phenotypes. Exposure to stresses which they encounter at various stages of their life cycles demands the ability to adapt. Consistent with this, many mycobacteria encode a multiplicity of genes for numerous important pathways such as respiration and cofactor biosynthesis [79,80], which allows for a more nuanced regulation of physiology. The analysis performed herein summarises the general distribution of PG remodelling genes in diverse strains and reveals an emerging trend towards gene multiplicity in environmental mycobacteria. There is great conservation within the MTBC and other pathogenic mycobacteria. Of all strains, *M. gilvum* displays the greatest degree of gene expansion, containing a total 44 PG remodelling genes, Table 1. This organism has not been studied extensively but may represent a potential model system for understanding how the genetic multiplicity for PG remodelling enzymes contributes to bacterial physiology. As expected *M. leprae* shows a reduction in the number of genes that encode the enzymes assessed in this study but still retains more than one representative of each functional class. This, together with the striking degree of conservation in some families of PG remodelling enzymes in pathogenic mycobacteria, suggests that PG biosynthesis, remodelling and possibly recycling are all potential vulnerable pathways for drug development. The extracellular nature of these enzymes provides an added advantage for drug screening since small molecules need not enter the cell for biological activity. Entry of compounds into mycobacterial cells remains the major confounding factor in current drug development initiatives. Moreover, the lack of human counterparts would ensure a high degree of specificity. In conclusion, the gene complements for PG remodelling revealed in this study most likely reflect the differential requirements of various mycobacteria for murein expansion/turnover during colonisation of and proliferation within host organisms or environmental niches.

Methods
The 19 mycobacterial strain sequences used in this study were all complete and either published [24,78,81-90] or directly submitted to GenBank [91] (Additional file 2: Table S1). The following sites were utilized for analysis of the genomes (Additional file 2: Table S2): The comparative genomic profile for the enzymes of interest were initiated by homology searches of known *M. tuberculosis* H37Rv genes at TubercuList [92], GenoList [93] or TBDB [94].
Where necessary for further analysis direct BLAST analysis was performed at NCBI [95], utilising protein sequence for BLASTp or DNA sequence for BLASTn particularly for the analysis of Mycobacterium sp. JLS, M. afric平um and M. intracellulare which are not or only partially annotated at TDBD. To confirm the absence of genes, protein sequence was used for tBLASTn analysis. Additional homologues that are absent from M. tuberculosis H37Rv were identified by advanced search at SmegmaList (Mycobrowser) [96]. Where information was required for sequence level analysis, the Sanger Artemis Comparison Tool (ACT) [97] was utilized on annotated sequences obtained from the Integrated Microbial Genomes (IMG) site at the DOE Joint Genome Institute [98]. Phylogeny was established from FASTA files from all genes in Table 1 at EMBL-EBI by Clustalo [99] alignment and ClustalW2 [100] analysis and visualized using FigTree V1.4 software (http://tree.bio.ed.ac.uk/software/figtree). Functional annotation of each of the M. tuberculosis genes was identified at InterScanPro [101], for PFAM domains [102], signal sequences (SignalP) [103] and membrane anchoring domains (TMHMM) [104].

Additional files

Additional file 1: Figure S1. Phylogenetic relationship between Resuscitation Promoting Factors from various mycobacteria. Figure S2 Phylogenetic relationship between Class A penicillin binding proteins (PonA family) from various mycobacteria. Figure S3 Phylogenetic relationship between Class B penicillin binding proteins (Pbp family) from various mycobacteria. Figure S4 Phylogenetic relationship between Class C penicillin binding proteins (DD-carboxypeptidases) from various mycobacteria. Figure S5 Phylogenetic relationship between endopeptidases (Nlp/Pgp domain containing proteins) from various mycobacteria. Figure S6 Phylogenetic relationship between LD-transepitidases from various mycobacteria. Figure S7 Phylogenetic relationship between N-acetylmyrumoyl-L-alanine from various mycobacteria.

Additional file 2: Table S1. Mycobacterial strains included in this study. Table S2. Bioinformatics sites used for analysis.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
BDK conceived and designed the study, EEM conducted all the bioinformatics analyses and compiled the manuscript. SS and CE provided intellectual input on certain aspects of the study. All authors approve of the final content in the manuscript.

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