Identification of the *namH* Gene, Encoding the Hydroxylase Responsible for the *N*-Glycolylation of the Mycobacterial Peptidoglycan*

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The peptidoglycan of most bacteria consists of a repeating disaccharide unit of β-1,4-linked *N*-acytylmuramic acid and *N*-acyetylg glucosamine. However, the muramic acid moieties of the mycobacterial peptidoglycan are *N*-glycolylated, not *N*-acyetylated. This is a rare modification seen only in the peptidoglycan of mycobacteria and five other closely related genera of bacteria. The *N*-glycolylation of sialic acids is a unique carbohydrate modification that has been studied extensively in eukaryotes. However, the significance of the *N*-glycolylation of bacterial peptidoglycan is unknown. The goal of this project was to identify the gene encoding the hydroxylase responsible for the *N*-glycolylation of the mycobacterial peptidoglycan. We developed a novel assay for the mycobacterial UDP-β-N-acetylmuramic acid hydroxylase by computer database searching and motif comparisons with the eukaryotic enzymes responsible for the *N*-glycolylation of sialic acids. The *namH* gene is not essential for *in vitro* growth as we were successful in deleting the gene in *M. smegmatis*. The *M. smegmatis* mutant is devoid of UDP-β-N-acetylmuramic acid hydroxylase activity and synthesizes only *N*-acyetylated muropeptide precursors. Furthermore, the mutant exhibits increased susceptibility to β-lactam antibiotics and lysozyme. Our studies suggest that the *N*-glycolylation of mycobacterial peptidoglycan may play a role in lysozyme resistance or may contribute to the structural stability of the cell wall architecture.

In a manner similar to the outer membrane of Gram-negative bacteria, the outer portion of the cell envelope of mycobacteria is believed to be arranged as an asymmetric bilayer, with an outer layer composed of various lipids with short to medium length fatty acids, and an inner layer composed of long chain mycolic acids covalently attached to the arabinogalactan polysaccharide (1, 2). The arabinogalatan is in turn covalently attached to the peptidoglycan via an essential rhamnose-*N*-acyetylg glucosamine linker, forming the mycolylarabinogalactan-peptidoglycan complex (mAGP) (3, 4). The peptidoglycan ultimately serves to anchor the principle mass of the mycobacterial cell envelope.

Peptidoglycan is an essential component of the cell envelope of virtually all bacteria, providing both shape and structural integrity to the cell. Peptidoglycan fragments are also biologically active molecules with toxicogenic and immunomodulatory properties (5–8). In general, peptidoglycan is comprised of a β-1,4-linked polymer of *N*-acyetylated muramic acid and glucosamine carbohydrates with cross-linked peptides of varying composition attached to the muramyl moieties. In virtually all bacteria, both carbohydrates in the glycan chain are *N*-acyetylated, however, in the mycobacteria the muramic acid moieties are *N*-glycolylated instead (9). Only five other genera of bacteria, *Rhodococcus*, *Tsukamurella*, *Gordonia*, *Noardia*, and *M. micronospora* have *N*-glycolyrmuramic acid in their peptidoglycan (10). These five genera are closely related to mycobacteria, and all six belong to the class Actinomyctetales. Why *N*-glycolylated muramic acid is only produced by a small number of bacteria is unknown. *N*-glycolylation is a rare carbohydrate modification seen only for muramic acid in these actinobacteria and for neuraminic acids (sialic acids) in eukaryotes (11). The *N*-glycolylation of sialic acids in eukaryotes is known to confer differential biological activities upon the carbohydrates. However, it is not known what role *N*-glycolyrmuramic acid plays in peptidoglycan biology, although it has been hypothesized to stabilize the cell wall through hydrogen bonding (2).

The initial discovery of *N*-glycolylated muramic acid (*MurNGlyc*) in mycobacteria prompted the hypothesis that it was synthesized from an *N*-acyetylated muramic acid (*MurNac*) precursor by the action of a monoxygenase enzyme, known generally as a hydroxylase (12). These enzymes typically require a proton donor, usually NADH or NADPH, for the reduction of molecular oxygen to water prior to the insertion of an oxygen atom into the substrate and forming a hydroxyl group (13). Previous work with *Mycobacterium phlei* established that the production of *N*-glycolyrmuramic acid in mycobacteria likely occurs by the action of an atmospheric oxygen-dependent hydroxylase.

The abbreviations used are: MurNGlyc, *N*-glycolylated muramic acid; MurNac, *N*-acyetylated muramic acid; MOPS, (3-(N-morpholino)propanesulfonic acid); HPLC, high pressure liquid chromatography; namH, *N*-glycolyrmuramic acid hydroxylase; LB, Luria-Bertani medium; MIC, minimum inhibitory concentration; MS, mass spectrometry.

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that converts UDP-MurNAc to UDP-MurN-Glyc (14). In addition, a soluble activity present in Nocardia asteroides capable of converting UDP-MurNAc to UDP-MurN-Glyc in an NADPH-dependent fashion was demonstrated almost 30 years ago (15), but since then no UDP-MurNAc hydrolysis has been purified and no gene identified from any bacterial species.

Our laboratory studies the biosynthesis of the mycobacterial peptidoglycan and we are particularly interested in determining the significance of N-glycolylmuramic acid in the cell wall of these organisms. In this study, we report the development of a new assay to detect the conversion of UDP-MurNAc to UDP-MurN-Glyc and the identification of the namH gene (N-acetylglucosamine acid hydroxylase) encoding the mycobacterial UDP-MurNAc hydroxylase. We also show that while the namH gene is not essential for the survival of Mycobacterium smegmatis, a namH deletion mutant is hypersusceptible to β-lactam antibiotics and lysozyme.

**Experimental Procedures**

**Bacterial Strains, Culture Techniques, Electrotoporation of M. smegmatis, and Reagents—** M. smegmatis strains mc155 (esp-1) (16), mc1255 (esp-1, rpsL4; epl41) (17), PM965 (esp-1 rpsL4 slbA1), and PM979 (esp-1 rpsL4 slbA1 ΔnamH1) were grown in LBT (Luria-Bertani broth supplemented with 0.5% (v/v) Tween 80, 0.05% (v/v) glycerol), or on LTF agar, or on Middlebrook 7H9 medium containing ADS (0.5% bovine serum albumin fraction V (Roche Applied Science), 0.2% glucose, 0.85% NaCl), 0.05% (v/v) Tween-80, 0.2% (v/v) glycerol), or on Middlebrook 7H10 agar supplemented with ADS, Tween-80, and glycerol. The slbA1 deletion in PM965 and PM979 removes the major β-lactamase enzyme of M. smegmatis and allows us to test the β-lactam susceptibilities of the strains in this study (18). Escherichia coli K-12 DH10B (MDH-Acetyl/RMS-mcrBC) slb0sla2AM15 ΔlacX74 deoR acr1 endA1 araΔ139 Δ(muc, leu) 7697 galU galK λ-pils (Str1) (Invitrogen, Carlsbad, CA) was used for plasmid DNA cloning, and E. coli strains were grown on LB agar or in LB medium. All strains were incubated at 37 °C. When necessary, the experiment was performed at 37 °C for 15 min at which time protein was removed from the reactions using an Amicon Centricon YM-10 filter device (Millipore Corp., Bedford, MA) by centrifugation at 7,500 × g for 1 h at 4 °C. Following protein removal, 10 μl of each sample was added to 150 μl of 0.1% (v/v) absolute ethanol and allowed to react for 4 h. The clear supernatant was saved at 4 °C.

**Preparation of M. smegmatis Whole Cell Extracts—** M. smegmatis lysozyme was added to 1 ml of the supernatant (2 mg of total protein/reaction) for a final reaction volume of 300 μl. Reactions were incubated at 37 °C for 15 min at which time protein was removed from the reactions using an Amicon Centricon YM-10 filter device (Millipore Corp., Bedford, MA) by centrifugation at 7,500 × g for 1 h at 4 °C. Following protein removal, 10 μl of each sample was added to 150 μl of 0.1% (v/v) absolute ethanol and allowed to react for 4 h. The clear supernatant was saved at 4 °C.

**UDP-MurNAc Hydroxylase Assay—** UDP-MurNAc hydroxylase reactions contained UDP-MurNAc[14C]Ac (2 × 10⁹ Bq/mm), (1 nmol), β-NADPH (250 nmol) in 50 mM Tris pH 8.0 buffer with 1 mM dithiothreitol. Mixtures were prewarmed to 37 °C, and reactions were initiated by the addition of a freshly prepared 1:1 slbA1/whole cell extract, soluble extract, or membrane fraction (2 mg of total protein/reaction) for a final reaction volume of 300 μl. Reactions were incubated at 37 °C for 15 min at which time protein was removed from the reactions using an Amicon Centricon YM-10 filter device (Millipore Corp., Bedford, MA) by centrifugation at 7,500 × g for 1 h at 4 °C. Following protein removal, 10 μl of each sample was added to 150 μl of 0.1% (v/v) absolute ethanol and allowed to react for 4 h. The clear supernatant was saved at 4 °C.

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derivatized products were dissolved in a small volume of hexane and analyzed in a ThermoQuest Trace GC 2000 gas chromatograph linked to a Finnigan Polaris mass detector.

**M. smegmatis Antibiotic and Lysozyme Susceptibility Assays—** Determination of antibiotic susceptibility was done by disk diffusion assays, while lysozyme susceptibilities were assayed by minimal inhibitory concentration tests using the macrodilution method as previously described (18). Minimum inhibitory concentrations (MIC) of lysozyme were noted as the lowest concentration at which no microbial growth could be observed or a noticeable growth defect (granular clumping) had occurred.

**M. smegmatis Lysozyme Growth Curves—** M. smegmatis strains were grown to saturation in 7H9 broth and then diluted 10-fold into fresh 7H9 medium and incubated at 37 °C. Optical density readings were recorded at 30-min intervals. Upon reaching an OD$_{600}$ of 0.4, the cultures were split into two, and freshly prepared lysozyme was added to a final concentration of 20 μg/ml to one of each of the split cultures of each strain. Readings were continued at 30-min intervals until each culture reached an OD$_{600}$ of 1.0.

**RESULTS**

**Development of the UDP-MurNAc Hydroxylase Assay—** UDP-MurNAc hydroxylase activity was first described in the actinobacterial species *N. asteroides* nearly 30 years ago using an assay that monitored the NADPH-dependent conversion of radiolabeled UDP-MurNAc to UDP-MurNGlyc (15). The substrate and product were detected using paper chromatography with comparison to chemically synthesized standards. Unfortunately, this assay was difficult for us to reproduce without UDP-MurNAc and UDP-MurNGlyc for comparison, as neither of these compounds is commercially available. Therefore, we decided to develop a new, HPLC-based assay, for the detection of the mycobacterial UDP-MurNAc hydroxylase activity, using UDP-MurNAc synthesized in our laboratory from commercially available UDP-GlcNAc.

Our initial attempts to assay the hydroxylase using UDP-MurNAc or radiolabeled UDP-MurNAc synthesized from UDP-GlcNAc in which the hexosamine backbone was $^{14}$C-labeled, were unsuccessful (data not shown). In each case, we were not able to unambiguously determine if UDP-MurNGlyc was produced in our assays, as we had no authentic UDP-MurNGlyc available for comparison. To overcome this problem, we used UDP-MurNAc in which the acetyl group of the nucleotide sugar was $^{14}$C-labeled in order to facilitate the identification of UDP-MurNGlyc ("Experimental Procedures"). The detection method is based upon the observation that acid hydrolysis of N-acetylated hexosamine sugars liberates the acyl group as a free acid. Therefore, acid hydrolysis of the hydroxylase assay reaction mixtures would release the labeled acyl groups of UDP-MurNAc and UDP-MurNGlyc as $^{14}$C-acetic acid and $^{14}$C-glycolic acid, respectively. These acids are easily distinguished from each other by their separation profiles in anion exchange HPLC.

A schematic representation of our mycobacterial UDP-MurNAc hydroxylase assay is shown in Fig. 1. Completed reaction mixtures were clarified using an Amicon filtration device, and then subjected to acid hydrolysis. The components of the hydrolysate were separated by anion exchange HPLC for 15 min, and the fractions containing the $^{14}$C organic acids were detected by scintillation counting. The radioactive peaks were compared with our established retention times for unlabeled glycolic acid (9.5 min) and unlabeled acetic acid (10.8 min).
standards. Because the acid hydrolysis is essentially 100% efficient in the removal of acyl groups from nucleotide sugars (data not shown) the amount of 14C-glycolic acid detected after acid hydrolysis of the reaction components corresponds to the amount of UDP-MurN Glyc produced.

The UDP-MurNAc hydroxylase activity in reactions containing 2 mg of protein of a whole cell extract of wild-type M. smegmatis strain PM965 was 23 pmol of UDP-MurN Glyc/min/mg protein (Table I). The activity was present in a soluble fraction prepared from the cell extract but was not present in the membrane fraction (Table I). The addition of a 10-fold excess of unlabeled UDP-MurNAc to reactions with whole cell extracts decreased the amount of labeled product by a factor of ten. However, the same result was not seen when a 10-fold excess of unlabeled UDP-GlcNac was added to the reactions (Table I).

Identification of the Bacterial UDP-MurNAc Hydroxylase Genes—Only two carbohydrate N-glycolylation reactions have been demonstrated in biology: the N-glycolylation of CMP-N-acyteluramamic acid in many eukaryotic organisms and the N-glycolylation of UDP-N-acytelurammamic acid in a limited number of bacteria. We used the protein sequence of the mouse CMP-N-acytelurammamic acid hydroxylase in a BLAST search (www.ncbi.nlm.nih.gov/BLAST/) for a homolog in the sequenced and annotated Mycobacterium tuberculosis H37Rv genome (24). The best match that we obtained was open reading frame Rv3818. Additional BLAST searches revealed the presence of Rv3818 homologs within the genomes of all the available mycobacterial species (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) including M. smegmatis, Mycobacterium avium, and Mycobacterium leprae, in which the gene is designated an pseudogene in the annotated genome, (25) as well as the homologous open reading frame present within M. smegmatis the bacterial UDP-MurNAc hydroxylases. We propose to name the corresponding gene namH.

The namH Gene Is Not Essential to M. smegmatis—We tested the essentiality of the namH gene in M. smegmatis by allelic exchange. We constructed a sacB-based suicide plasmid, pMP354, bearing an unmarked, 500-bp deletion allele (∆namH1). The deletion of the gene in wild-type M. smegmatis strain PM965 was done by a previously described counterselection protocol (“Experimental Procedures”). Southern blot analysis confirmed the exchange of the ∆namH1 allele with that of wild type in the M. smegmatis genome of strain PM979 (data not shown).

The M. smegmatis ∆namH1 Mutant Is Devoid of UDP-MurNAc Hydroxylase Activity—No UDP-MurNAc hydroxylase activity was observed in reactions containing 2 mg of protein of whole cell extracts prepared from the ∆namH1 mutant (Table I). Complementation of the mutant with a plasmid bearing the wild-type gene restored UDP-MurNAc hydroxylase activity to levels greater than that seen within the parental strain (Table I). The increased hydroxylase activity of the complemented mutant compared with the parental strain can be attributed to the use of a multicopy plasmid containing a promoter that may be stronger than the native namH promoter.

The M. smegmatis ∆namH1 Mutant Lacks N-Glycolylated Peptidoglycan Precursors—We isolated and analyzed the peptidoglycan precursor muropeptidpeptides from the wild-type parental strain PM965 and the ∆namH1 mutant PM979 by LC-MS to determine the acylation state of the muramyl moiety in each sample. The positive ion mass spectra of the muropeptides from PM965 (namH+) were dominated by ions having m/z values of 824.2 and 808.2 in an apparent ratio of 7:3 (Fig. 3). These ions have been previously identified as MurN Glyc-t-Ala-d-Glu-DAP-t-Ala-α-Ala and MurN Ac-t-Ala-d-Glu-DAP-t-Ala-α-Ala, respectively (22). In contrast, the positive ion mass spectra of the muropeptides from PM979 (∆namH1) were dominated by an ion having an m/z value of 808.2 and the corresponding sodium adduct (m/z 830.3), consistent with the muropeptide MurN Ac-t-Ala-d-Glu-DAP-t-Ala-α-Ala. No signal consistent with MurN Glyc-t-Ala-d-Glu-DAP-t-Ala-α-Ala was observed from muropeptides purified from the ∆namH1 mutant. The identities of muramyl residues in the purified muropeptides from both strains were confirmed by GC-MS analysis after

### Table I

**Mycobacterial Peptidoglycan Hydroxylase Enzymatic activity**

| Reaction         | Parental | ∆namH1 | ∆namH1/∆namH* |
|------------------|----------|--------|---------------|
| Whole cell extract | 23       | 0      | 32            |
| Soluble fraction  | 28       | 0      | ND            |
| Membrane fraction | 0        | ND     | ND            |

| Reaction         | Parental | ∆namH1 | ∆namH1/∆namH* |
|------------------|----------|--------|---------------|
| + 10nmol UDP-GlcNac | 19      | ND     | ND            |
| + 10nmol UDP-MurNAc | 2       | ND     | ND            |

* ND, not determined.

### Table II

**Comparison of actinobacterial UDP-MurNAc hydroxylases**

| Organism | M. smegmatis | M. tuberculosis | M. avium | N. farcinica |
|----------|--------------|----------------|----------|-------------|
| R. sp RHA1 | 77           | 76             | 76       | 86          |
| N. farcinica | 78           | 77             | 76       | –           |
| M. avium   | 82           | 87             | –        | –           |
| M. tuberculosis | 82       | –             | –        | –           |
The M. smegmatis ΔnamH1 Mutant Is Hypersusceptible to β-Lactam Antibiotics and Lysozyme—Although namH was determined to be a non-essential gene for M. smegmatis, we hypothesized that the lack of N-glycolylated muramic acid in the peptidoglycan would have a detrimental effect on cell wall biosynthesis or stability. This hypothesis was evaluated by testing susceptibility of the ΔnamH1 mutant to various antibotics that interfere with cell envelope biosynthesis and to lysozyme.

We tested the sensitivity of the ΔnamH1 mutant to the β-lactam antibiotics amoxicillin and ampicillin, drugs that interfere with peptidoglycan assembly and cross-linking, by a disk diffusion assay. As shown in Table III, the mutant was hypersusceptible to β-lactams compared with the parental and complemented strains regardless of the media (rich or minimal) used for culture. However, the susceptibility of the mutant to amoxicillin (and to a lesser extent, ampicillin) was exacerbated when grown on rich medium (LB) as compared with minimal medium (7H9) (Table III). We observed no difference in susceptibility between the ΔnamH1 mutant and the parental and complemented strains to the antimycobacterial drugs ethambutol and isoniazid that target the biosynthesis of mycolic acids and arabinan, two other components of the mycobacterial cell envelope (Table III).

In this work, we demonstrated hypersusceptibility of the ΔnamH1 mutant to penicillin type β-lactam antibiotics. In addition, a namH mutant of M. smegmatis is also hypersusceptible to the cephalosporin type β-lactam ceftriaxone. In a separate project, our laboratory isolated a namH::mariner transposon insertion mutant of M. smegmatis from a transposon mutagenesis search for mutants hypersusceptible to cephalosporins (28). The phenotype of this mutant is due to the transposon insertion in namH as a plasmid bearing a copy of the wild-type namH gene confers parental ceftriaxone susceptibility to the mutant (data not shown).
A characteristic of organisms belonging to the genus *Mycobacterium* is an inherent resistance to lysozyme, a muramidase that cleaves the glycosidic bond between the sugar residues of the peptidoglycan chain. To see if the glycolylation state of the peptidoglycan affects lysozyme susceptibility, we determined the lysozyme MIC of the \( \text{H9004 namH1} \) mutant grown in both minimal and rich media. As shown in Table IV, both the wild-type and complemented strains were able to tolerate lysozyme challenge to a greater extent than the mutant strain, although all strains were more susceptible growing in minimal medium compared with rich. The fold difference in lysozyme MIC between the various strains tested was never greater than 2-fold when grown in minimal medium. However, the difference in lysozyme MIC between the *M. smegmatis* \( \text{H9004 namH1} \) mutant and controls strains was at least 8-fold when grown in rich medium (Table IV).

To further characterize the lysozyme sensitivity of the \( \text{H9004 namH1} \) mutant, we studied the effect of lysozyme added to...
mid-exponential phase cultures growing in minimal medium. The optical density of the cultures was recorded over an 8-h period following challenge with 20 μg/ml lysozyme (Fig. 4). Consistent with the lysozyme MIC results, growth of the mutant was hindered following the addition of lysozyme to the media. The optical density of the wild type and the complemented cultures plateaued at 0.5 h after addition of lysozyme. This plateau lasted for 2 h, after which the density of the cultures increased. In contrast, the density of the ΔnamH1 culture decreased 1 h after the addition of lysozyme and the cells appeared to begin to aggregate. The culture eventually recovered 3.5 h later, but the density of the ΔnamH1 cultures never matched that of the controls. The viable counts of the ΔnamH1 cultures did not decrease throughout the growth curve following lysozyme addition (data not shown). The recovery and continuation of culture density during later time points of the growth curve can be attributed to the lability of the enzyme, as addition of fresh lysozyme at these later time points affected the growth of the mutant (data not shown).

**DISCUSSION**

The occurrence of N-glycolylated muramic acid in peptidoglycan is very rare and the purpose of this modification is unclear. The presence of this sugar in only closely related members of the class Actinomycetales suggests a correlation between the presence of N-glycolylmuramic acid and the type of cell envelope in the organism. All of the N-glycolylmuramic acid-containing bacteria also have a mycolylarabinogalactan-peptidoglycan type of cell envelope. In this study we have identified the namH gene encoding the enzyme responsible for the production of N-glycolyl muramic acid in the mycobacterial peptidoglycan. This gene is found in all mycobacterial genomes sequenced to date, but it is a pseudogene in *M. leprae*. This was initially surprising, as the peptidoglycan of *M. leprae* has been reported to contain N-glycolylmuramic acid (29). A more recent study demonstrated that the peptidoglycan of *M. leprae* is not N-glycolylated (30), consistent with the identity of NamH as the enzyme responsible for this modification of the mycobacterial peptidoglycan. Given that the eukaryotic sialic acid hydroxylases require a cytochrome and cytochrome reductase for activity (11, 47, 48), there is the slight possibility that the namH gene encodes a protein that indirectly interacts with the peptidoglycan hydroxylase system in mycobacteria. To address this issue, we have made several attempts to express and purify NamH fusion proteins bearing N- or C-terminal polyhistidine or thioredoxin tags and then assay the purified protein with or without reconstitution using cell extract. Unfortunately, the NamH protein does not appear to tolerate fusions, as all of our fusion constructs failed to complement the namH deletion mutant. Likewise, attempts to overexpress the native protein in *E. coli* were unfruitful, since the protein is rather toxic to *E. coli* resulting in low protein expression or the generation of mutant namH genes (data not shown).

We show that namH is not essential for the growth of *M. smegmatis*, however, a namH deletion mutant is hypersusceptible to β-lactam antibiotics that interfere with peptidoglycan cross-linking and lysozyme. These two phenotypes could suggest that the N-glycolyl group does indeed serve to stabilize the cell wall via hydrogen bonds as previously suggested, and the loss of this group decreases the integrity of the wall such that β-lactam antibiotics and lysozyme have a greater ability to affect the peptidoglycan. However, there is very little information about the three-dimensional organization of this part of the mycobacterial cell wall. The rhamnose-N-acetylgalcosamine linker that connects the arabinan polysaccharide to the peptidoglycan is attached to the C6 of muramic acid via a phosphoryl group (3). It may be that the phosphoryl groups are involved in hydrogen bonding to the N-glycolylmuramic acid on adjacent strands, but not all of the muramic acid groups are substituted with the linker. If there were a broad defect in cell envelope stability in the PG of the ΔnamH1 mutant, it would be expected to have a generalized effect upon other aspects of the cell envelope, presumably resulting in hypersusceptibility to other antibiotics (i.e. isoniazid, ethambutol) that target the biosynthesis of other cell envelope components. However, the mutant has wild-type susceptibility to these antibiotics.

We favor an alternative hypothesis that the two phenotypes of the ΔnamH1 mutant are the result of separate mechanisms. The increased susceptibility to β-lactams is due to problems with either the translocation of the peptidoglycan precursors across the cytoplasmic membrane, the polymerization of the

**FIG. 4. Lysozyme growth curves.** The parental, ΔnamH1 mutant, and the complemented strains were grown in 7H9 medium in the absence (A) and presence (B) of lysozyme at a concentration of 20 μg/ml. Lysozyme was added to cultures at an OD₆₀₀ of ~0.4, and OD readings were recorded at 30-min intervals.
glycan backbone, or the recognition of the precursors by the peptidases responsible for cross-linking of the peptidoglycan peptides. We propose that the recognition of the abnormal sugar by the transglycosylases is affected. This could influence peptide cross-linking, as transpeptidation reactions are generally linked to the polymerization of the glycan chain. The net result of this would be a decrease in the amount of peptidoglycan cross-linking and thus, hypersusceptibility to β-lactam antibiotics. The second part of this hypothesis posits that the increased susceptibility of the ΔnamH1 mutant to lysozyme is because the specific function of the N-glycolyl group is to block the enzyme from the β-1,4 bond between the muramyl and the N-acetylglucosaminyl residues in the glycan chain. It is known that O-acetylation at C6 of the muramic acid ring confers lysozyme resistance (31), as does the de-acyetylation of N-acetylglucosamine (32). In this model, the replacement of the N-glycolyl group with N-acetyl on muramic acid in the ΔnamH1 mutant would result in a peptidoglycan with increased sensitivity to lysozyme.

The large differences in lysozyme sensitivity observed between the ΔnamH1 mutant and the parental strain during growth in rich versus minimal medium are interesting and ought to be explored in greater detail. All the strains were consistently more sensitive to lysozyme when grown in minimal medium. However, the fold difference in sensitivity between the parental strain and ΔnamH1 mutant were significantly more pronounced when grown in rich medium. Likewise, the mutant was more susceptible to the β-lactam antibiotic amoxicillin when grown in rich medium versus minimal medium. Whether or not these results are the consequence of metabolic changes under different growth conditions that affect the structure and permeability of the cell envelope remains to be determined.

Another question is whether this unique modification has a role in mycobacterial pathogenesis. Mammals possess a series of antibacterial enzymes (lysozymes, amidases) capable of cleaving bacterial peptidoglycan. Any modification of the peptidoglycan that confers resistance to these types of enzyme could have a role in pathogenesis. For Streptococcus pneumoniae, resistance to lysozyme is a virulence trait. This organism possesses a peptidoglycan de-acetylase that removes the N-acetyl group from N-acetylglucosamine, rendering the peptidoglycan lysozyme resistant (32). A S. pneumoniae mutant unable to de-acylate its peptidoglycan is sensitive to lysozyme and is attenuated in a mouse model (33). The N-glycolylation of the mycobacterial PG could play a similar role in M. tuberculosis virulence.

Peptidoglycan fragments are pathogen-associated molecular patterns recognized by the innate immune system (8). It is possible that N-glycolylated muramic acid could function to modulate the innate immune response to mycobacterial peptidoglycan fragments released during a mycobacterial infection. Recent studies demonstrated that the peptidoglycan of M. tuberculosis is 40 times more effective than E. coli peptidoglycan at inhibiting the interferon-γ induction of MHC Class II expression on macrophages (34). Given that the structures of the peptidoglycan of these two bacteria differ primarily on the basis of the muramic acid residues, we hypothesize that the presence of the N-glycolyl group on muramic acid in the mycobacterial peptidoglycan could explain the potency of M. tuberculosis peptidoglycan in effecting macrophage function.

REFERENCES

1. Brennan, P. J. (2003) Tuberculosis (Edinb) 83, 91–97
2. Brennan, P. J., and Nikaido, H. (1995) Annu. Rev. Biochem. 64, 29–63
3. Crick, D. C., Mahapatra, S., and Brennan, P. J. (2001) Glycobiology 11, 1073–111R
4. Yagi, T., Mahapatra, S., Mikusova, K., Crick, D. C., and Brennan, P. J. (2003) J. Biol. Chem. 278, 26497–26504
5. Burroughs, M., Rodzinski, E., Geelen, S., and Tuomanen, E. (1993) J. Clin. Investig. 92, 297–302
6. Luker, K. E., Tyler, A. N., Marshall, G. R., and Goldman, W. E. (1995) Mol. Microbiol. 16, 733–743
7. McIlvain, A. A., McGee, Z. A., and Rosenthal, R. S. (1984) J. Infect. Dis. 149, 378–386
8. Royet, J., and Reichhart, J. M. (2003) Trends Cell Biol. 13, 610–614
9. Azuma, I., Thomas, D. W., Adam, A., Ghuysen, J. M., Bonaly, R., Petit, F. J., and Lederer, E. (1970) Biochim. Biophys. Acta 209, 444–451
10. Holt, G. J., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (eds) (1994) Bergey’s Manual of Determinative Bacteriology, 9th edn. pp. 597–649, Williams & Wilkins, Baltimore
11. Angata, T., and Varki, A. (2002) Chem. Rev. 102, 439–469
12. Petrovich, J. F., Adam, A., and Wietzerbin-Falszpan, J. (1970) FEBS Lett. 6, 55–57
13. Mason, J. R., and Cammack, R. (1992) Annu. Rev. Microbiol. 46, 277–305
14. Essers, L., and Schoop, H. J. (1978) Biochim. Biophys. Acta 544, 180–184
15. Gateau, O., Bordet, C., and Michel, G. (1976) Biochim. Biophys. Acta 421, 395–405
16. Snapper, S. B., Molten, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R., Jr. (1990) Mol. Microbiol. 4, 1911–1919
17. Mielewski, E., Weisbrod, T. R., Marcinkeviciene, J. A., Bittman, R., and Jacobs, W. R., Jr. (1998) J. Bacteriol. 180, 4259–4267
18. Flores, A. E., Parsons, L. M., and Pavella, M. S., Jr. (2004) Microbiology, in press
19. Pavella, M. S., Jr., and Jacobs, W. R., Jr. (1996) J. Bacteriol. 178, 6496–6507
20. Pavella, M. S., Jr., and Jacobs, W. R., Jr. (1999) J. Bacteriol. 181, 4780–4789
21. Raymond, J. B., Price, N. P., and Pavella, M. S., Jr. (2005) FEBS Microbiol. Lett. 229, 83–89
22. Mahapatra, S., Scherman, H., Brennan, P. J., and Crick, D. C. (2004)
23. Alleschel, S. F., Gah, W., Miller, W., Myers, E. W., and Lipman, D. J. (1999) J. Mol. Biol. 215, 403–410
24. Cole, S. T., Brosh, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, R., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Felstow, T., Gentles, S., Hanlin, N., Holroyd, S., Hornsby, T., Jagels, K., Kruger, A., Mclaren, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squires, R., Squires, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrett, B. G. (1998) Nature 393, 537–544
25. Cole, S. T., Eiglmeier, R., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Parkhill, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squires, R., Squires, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrett, B. G. (1998) Nature 393, 537–544
26. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
27. Schlenzka, D., Shaw, L., Kelm, S., Schmidt, C. L., Bil, E., Trautwein, A. X., Lottspeich, F., and Schauer, R. (1996) FEBS Lett. 385, 197–200
28. Velzy, K., Flores, A. E., Speese, J. M., and Pavella, M. S., Jr. (2004)
29. Draper, P., Kandler, O., and Darbre, A. (1987) 133, 1187–1184
30. Mahapatra, S., and Crick, D. C. (2004)
31. Heckels, J. E., and Virji, M. (1988) in Bacterial Cell Surface Techniques (Hancock, I., and Poxton, I., eds) pp. 82–83, Wiley-Interscience, Bath, Avon
32. Vollmer, W., and Tomasz, A. (2000) J. Biol. Chem. 275, 20496–20501
33. Vollmer, W., and Tomasz, A. (2002) Infect. Immun. 70, 7176–7178
34. Kurune, S. M., Solache, A., Jaeger, A., Hill, P. J., Rehbein, J. T., Blem, B. R., Rubin, K. J., and Ernst, J. D. (2004) J. Immunol. 172, 6272–6280
35. Geer, L. Y., Domrachew, M., Lipman, D. J., and Bryant, S. H. (2002) Genome Res. 12, 1619–1623
