Defects in glycopeptidolipid biosynthesis confer phage I3 resistance in *Mycobacterium smegmatis*

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Mycobacteriophages have played an important role in the development of genetic tools and diagnostics for pathogenic mycobacteria, including *Mycobacterium tuberculosis*. However, despite the isolation of numerous phages that infect mycobacteria, the mechanisms of mycobacteriophage infection remain poorly understood, and knowledge about phage receptors is minimal. In an effort to identify the receptor for phage I3, we screened a library of *Mycobacterium smegmatis* transposon mutants for phage-resistant strains. All four phage I3-resistant mutants isolated were found to have transposon insertions in genes located in a cluster involved in the biosynthesis of the cell-wall-associated glycopeptidolipid (GPL), and consequently the mutants did not synthesize GPLs. The loss of GPLs correlated specifically with phage I3 resistance, as all mutants retained sensitivity to two other mycobacteriophages: D29 and Bxz1. In order to define the minimal receptor for phage I3, we then tested the phage sensitivity of previously described GPL-deficient mutants of *M. smegmatis* that accumulate biosynthesis intermediates of GPLs. The results indicated that, while the removal of most sugar residues from the fatty acyl tetrapeptide (FATP) core of GPL did not affect sensitivity to phage I3, a single methylated rhamnose, transferred by the rhamnosyltransferase Gtf2 to the FATP core, was critical for phage binding.

INTRODUCTION

Phages that infect mycobacteria have played an important role in the development of genetic tools for mycobacteria, particularly *Mycobacterium tuberculosis*, which is the causative agent of tuberculosis (Bardarov et al., 1997, 2002; Jacobs et al., 1991; Lee et al., 2004; Raj & Ramakrishnan, 1970; Snapper et al., 1988; van Kessel et al., 2008). Recombinant mycobacteriophages have also been used for phage typing of clinical isolates (Kubica, 1982; Rado et al., 1975), in diagnostics (Carriere et al., 1997; Hazbon et al., 2003; Jacobs et al., 1993; McNerney & Traore, 2005; Piuri et al., 2009; Riska et al., 1999), and as therapeutic agents for mycobacterial diseases (Broxmeyer et al., 2002; Mankiewicz & Beland, 1964). For the further development of mycobacteriophages as genetic tools for manipulation of mycobacteria, and as diagnostic and therapeutic agents, it is necessary to understand the mechanism of interactions between mycobacteriophages and mycobacteria. Attachment of a phage by binding to its receptor on the mycobacterial cell surface is important for initiating infection, and the study of phage-resistant mutants defective in phage adsorption is a useful approach to identifying these receptors. While a number of cell wall components have been implicated in roles as phage receptors (Besra et al., 1994; Bisso et al., 1976; Dhariwal et al., 1986; Furuchi & Tokunaga, 1972), no defined mycobacteriophage-resistant mutant with a missing phage receptor has been characterized to date. The only report of a mycobacteriophage-resistant mutant describes the appearance of a new lipid species (rather than the disappearance of an existing component) in a *Mycobacterium smegmatis* transposon mutant that was resistant to phage D29 (Besra et al., 1994). A study by Barsom & Hatfull (1996) demonstrated that overexpression of the *M. smegmatis* gene *mpr* confers resistance to multiple mycobacteriophages, but the function of *mpr* remains unknown.

Phage receptors would be expected to be outer components of the bacterial cell wall, and thus phage-resistant mutants would also be an extremely useful resource for identification of biosynthetic pathways of mycobacterial cell wall components. Indeed, studies of interactions of bacteriophages with host cells have often led to discovery of bacterial cell surface receptors and transporters that play important roles in microbial physiology (Heller, 1992; Estrela et al., 1991; Tran et al., 1999).

Abbreviations: FATP, fatty acyl tetrapeptide; GPL, glycopeptidolipid.
Phage I3 is a generalized transducing phage belonging to the Myoviridae family. It infects *M. smegmatis*, and it has found utility as a genetic tool for generalized transduction (Raj & Ramakrishnan, 1970). In this study, we used phage-I3-resistant mutants of *M. smegmatis* isolated from a transposon library to define the receptor for phage I3, and to demonstrate the utility of phage-resistant mutants for identification of genes involved in the biosynthesis of mycobacterial cell wall components.

**METHODS**

**Bacterial strains, plasmids, phages and culture conditions.** Bacterial strains, phages and plasmids used in this study are listed in Table 1. *M. smegmatis* strains were grown at 37 °C in Middlebrook 7H9 broth or tryptic soy broth (TSB), or on Middlebrook 7H10 medium or TSB agar (Becton Dickinson). For selection of the phage, 7H9 broth or tryptic soy broth (TSB), or on Middlebrook 7H10 medium were used to construct transposon mutant libraries of *M. smegmatis* according to protocols described by Larsen *et al.* (2006). Bacterial strains to different phages was determined by spotting onto a 7H9 agar plate that contained an overlay of soft agar (7H9 medium or TSB agar pre-seeded with a suspension of I3 phage (10^10 p.f.u. per plate), mutants were pre-inoculated in 96-well plates, and then replicated in 96-well plates. Phage titres were prepared, and phage titres were determined, according to protocols described by Larsen *et al.* (2007). Sensitivity of *M. smegmatis* strains to different phages was determined by spotting 10 μl of a serial 10-fold dilution (10^−3–10^−7) of phage onto a 7H9 agar plate that contained an overlay of soft agar (7H9 +0.6 % agar) mixed with 100 μl mid-exponential culture of *M. smegmatis* mc^155^. Plates were incubated at 37 °C for 2 days to visualize plaques.

**Isolation of phage-I3-resistant mutants.** Temperature-sensitive transposon-delivery phages phAE185 (containing Tn5370; McAdam *et al.*, 2002) and phAE181 (containing Tn5371; Kriakov *et al.*, 2003) were used to construct transposon mutant libraries of *M. smegmatis* mc^155^, according to protocols described by Larsen *et al.* (2007). For isolation of phage-I3-resistant mutants, individual transposon mutants were pre-inoculated in 96-well plates, and then replicated with a 96-splice replicator onto rectangular plates containing TSB agar pre-seeded with a suspension of I3 phage (10^10^ p.f.u. per plate), to screen for strains resistant to killing by phage. Single colonies of each candidate strain were then cultured and re-tested for phage resistance.

**Detection of intracellular phage I3 DNA.** *M. smegmatis* strains, grown to an OD^600_ of 1.0, were washed with MP buffer [50 mM Tris/HCl (pH 7.8), 150 mM NaCl, 10 mM MgSO_4_, 2 mM CaCl_2], and incubated with phage I3 (m.o.i. 1) for 1 h at 37 °C. The suspension was then centrifuged at 3000 r.p.m. (MSE 43124-141 rotor) and the cell pellet was washed once in PBS, resuspended in 1 ml DNase I reaction buffer [20 mM Tris/HCl (pH 8.4), 2 mM MgCl_2_, 50 mM KCl] containing 3 units DNase I, and incubated for 20 min at 37 °C for digestion of any traces of phage DNA. DNase I was then inactivated by adding EDTA solution to a final concentration of 2 mM, and heating for 10 min at 65 °C. The cell pellet was washed four times with PBS containing 0.1 % Tween-80. Total DNA was then extracted from the washed cell pellet using standard protocols (Larsen *et al.*, 2007), and it was used as template for PCR detection of intracellular phage I3 DNA. The primers I317kD-F (5′-GTAC-AACCCGCCAAACCCAC-3′) and I317kD-R (5′-CAGGGGACG-AGATAGGTG-3′), designed to amplify part (461 bp) of a phage I3 gene encoding a 17 kDa structural protein (Ramesh & Gopinathan, 1994), were used for PCR amplification.

**Isolation and sequencing of transposon-insertion sites.** For all phage-resistant mutants, with the exception of 13R-1, BshIII-digested genomic DNA was used in a ligation reaction. To select for self-ligated fragments of genomic DNA containing Tn5371 (containing hyg and the R6K ori), the ligation mix was transformed into *E. coli* CC118/pir (Herrero *et al.*, 1990). Plasmid DNA isolated from hygromycin-resistant transformants was then sequenced using the primers Tn5371-L (5′-AGTGCCACCTAAATTGTAAGC-3′) and Tn5371-R (5′-TAGACAGATCGTGATAGAG-3′) to obtain sequences of the left and right regions, respectively, flanking the transposon insertion. For the mutant 13R-1, genomic DNA was digested with BamHI, and then ligated into BamHI-digested pUC19 (Yanisch-Perron *et al.*, 1985). Recombinant clones containing the transposon-insertion-containing genomic DNA fragment were isolated by selecting *E. coli* Top10 (Invitrogen) transformed with the reaction buffer and heating for 10 min at 65 °C. The cell pellet was washed four times with PBS containing 0.1 % Tween-80. Total DNA was then extracted from the washed cell pellet using standard protocols (Larsen *et al.*, 2007), and it was used as template for PCR detection of intracellular phage I3 DNA. The primers I317kD-F (5′-GTAC-AACCCGCCAAACCCAC-3′) and I317kD-R (5′-CAGGGGACG-AGATAGGTG-3′), designed to amplify part (461 bp) of a phage I3 gene encoding a 17 kDa structural protein (Ramesh & Gopinathan, 1994), were used for PCR amplification.

**Biochemical characterization of *M. smegmatis* strains.** Polar and apolar lipids (labelled with [14C]acetate) were extracted by using the methods described by Dobson *et al.* (1985). Glycopeptidolipids

<table>
<thead>
<tr>
<th>Strain or phage</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. smegmatis</strong></td>
<td>Parental strain</td>
<td>Snapper <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>mc^155^</td>
<td>Parental strain</td>
<td>Miyamoto <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Δgtf1</td>
<td>gtf1 disruptant of mc^155^</td>
<td>Miyamoto <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Δgtf2</td>
<td>gtf2 disruptant of mc^155^</td>
<td>Miyamoto <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Δgtf3</td>
<td>gtf3 disruptant of mc^155^</td>
<td>Miyamoto <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Δgtf2-pMV261gtf2</td>
<td>Δgtf2 complemented with a gtf2-containing plasmid</td>
<td>Miyamoto <em>et al.</em> (2006)</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td>Generalized transducing mycobacteriophage</td>
<td>Raj &amp; Ramakrishnan (1970)</td>
</tr>
<tr>
<td>I3</td>
<td>Generalized transducing mycobacteriophage</td>
<td>Froman <em>et al.</em> (1954)</td>
</tr>
<tr>
<td>Bx1</td>
<td>Generalized transducing mycobacteriophage</td>
<td>McAdam <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>phAE185</td>
<td>Temperature-sensitive derivative of mycobacteriophage TM4 containing the IS1096-derived transposon Tn5370</td>
<td>Kriakov <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>phAE181</td>
<td>Temperature-sensitive derivative of mycobacteriophage TM4 containing the Himar1-derived transposon Tn5371</td>
<td>Kriakov <em>et al.</em> (2003)</td>
</tr>
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(GPLs) from polar lipids were separated by TLC, following base treatment, as described previously (Burguiere et al., 2005). [14C]-labelled lipids on TLC plates were visualized after overnight exposure to Kodak X-Omat film.

RESULTS

Isolation of phage-I3-resistant mutants of M. smegmatis from a random transposon library

Libraries of M. smegmatis random transposon mutants, generated using either the IS1096-derived transposon Tn5370 (Cirillo et al., 1991; McAdam et al., 2002), or the Himar1-derived transposon Tn5371 (Kriakov et al., 2003), were screened for resistance to lysis by phage I3 by replicating 96-well plates containing transposon mutants on agar plates pre-seeded with phage I3. After single colony purification, the putative phage-resistant mutants were subjected to a second culture passage in the presence of phage to yield four bonafide phage-I3-resistant mutants (no plaques were observed when a suspension of phage I3 was spotted onto lawns of individual mutant strains; Fig. 1). Intracellular phage I3 genomic DNA was detected by PCR from M. smegmatis mc2155 cells pre-adsorbed with phage I3, but not from phage-resistant mutants, indicating that the phage failed to inject DNA into the phage-resistant mutants (Fig. 2).

Phage-I3-resistant M. smegmatis mutant strains are defective in GPL biosynthesis

Following the cloning of transposon-tagged genomic insertion sites from the phage-I3-resistant mutants in E. coli, the transposon-disrupted genes were identified by sequencing using transposon-specific primers (Table 2). All of four I3-resistant mutants were found to contain insertions that mapped to a cluster of genes involved in the biosynthesis of GPLs (Table 2). GPLs are a group of complex lipids derived from an acylated non-ribosomal tetrapeptide core and carbohydrate moieties, and they are abundant in the outer envelope of M. smegmatis and other mycobacteria (Billman-Jacobe, 2004; Schorey & Sweet, 2008). M. smegmatis produces six subclasses of GPLs, each of which has a common fatty acyl tetrapeptide (FATP) core consisting of a tetrapeptide amino alcohol (d-Phe-d-allo-Thr-d-Ala-l-alaninol) linked to a C26–C34 fatty acyl chain via an amide bond (Miyamoto et al., 2006; Fig. 3a). GPL-1, -2, -3 and -4 contain a rhamnose (with varying levels of O-methylation) and di-acetylated 6-deoxytalose as sugar residues. GPL-5 and -6 contain an additional O-methyl rhamnose attached to the original rhamnose residue (Miyamoto et al., 2006).

Given the association of the transposon insertions with genes involved in GPL biosynthesis, we conducted a thorough analysis of polar and apolar lipids from the phage-resistant mutant strains using 2D TLC (Dobson et al., 1985). The analysis revealed a loss of GPLs in all mutants (Fig. 4 b–e). None of the other cell-wall-associated lipids was altered in any of the mutants (data not shown). Additionally, TLC analysis used for separation of de-acetylated subclasses 1–4 of GPLs showed that these subclasses were absent in the mutant strains (Fig. 4f). These results suggested a role for GPLs in binding of phage I3 to the M. smegmatis cell surface.

Loss of GPLs does not result in resistance to other mycobacteriophages

Given the location of GPLs as one of the outer glycolipids of the M. smegmatis cell envelope, it was possible that...
Phage resistance was not due to loss of a phage I3 receptor, but due to a non-specific effect affecting adsorption of all mycobacteriophages. However, when we tested the sensitivity of the phage-I3-resistant mutants to two other lytic mycobacteriophages (D29 and Bxz1), both phages were able to form plaques on lawns of the phage-I3-resistant mutants, indicating that GPL deficiency caused resistance that was specific to phage I3 (Fig. 1). Thus, GPLs are likely to be the receptors for phage I3.

Minimal structural requirements for the phage I3 receptor

The sequence of assembly of sugar residues in GPLs was identified by Miyamoto et al. (2006) by specifically deleting gtf1, gtf2 or gtf3, which are three genes encoding glycosyltransferases in M. smegmatis mc^2155. Gtf1 is involved in the transfer of rhamnose to the FATP core, and consequently the Δgtf1 mutant lacked all classes of GPLs, and instead accumulated the intermediates FATP-3,4-di-O-methyl rhamnose and FATP-2,3,4-tri-O-methyl rhamnose (FATP-di-O-Me-Rha and FATP-tri-O-methyl-rhamnose; Fig. 3b, c). Similarly, Gtf2 is involved, independently, in the transfer of rhamnose to the FATP core, and the Δgtf2 mutant, which also lacked all classes of GPLs, accumulated FATP-6-deoxylactose. (Fig. 3d). On the other hand, Gtf3 is involved in the transfer of the second rhamnose residue, and subsequently only GPL-5 and -6 were missing in the Δgtf3 mutant. The accumulation of different intermediates of GPL biosynthesis in these mutants allowed us to utilize these strains to identify the specific structural components of GPL that defined the receptor of phage I3. The Δgtf1 and Δgtf3 mutants were sensitive to infection by phage I3, though the plaqueing efficiency of phage I3 on a lawn of the Δgtf1 mutant was diminished (Fig. 5). On the other hand, the Δgtf2 mutant was found to be phage resistant, and complementation of the Δgtf2 mutant with plasmid-encoded gtf2 restored phage sensitivity (Fig. 5). This was consistent with the phage-resistance phenotype of the transposon mutant I3R-1, which had a transposon insertion in gtf2 (Table 2). Also, resistance of the Δgtf2 mutant to phage I3 correlated with the inability to detect intracellular phage DNA by PCR following adsorption with phage (Fig. 2). Similar to the I3-resistant transposon mutants, the Δgtf2 mutant retained sensitivity to phages D29 and Bxz1 (Fig. 5). Thus, collectively, these results indicate that while the 6-deoxytalose residue is not essential for phage binding, addition of the first rhamnose residue to the FATP core is critical for phage I3 infection, and a mixture of FATP cores containing a di- or tri-O-methylated rhamnose is sufficient to allow binding of phage I3.

DISCUSSION

Most mycobacteriophages belong to the family Myoviridae or Siphoviridae; these families encompass phages with either contractile or long tails (Ackermann, 2001). It has been speculated that this tail morphology may be particularly relevant to mycobacteriophages, helping injection of phage DNA through an exceptionally thick, carbohydrate- and lipid-rich cell wall (Brennan & Nikaido, 1995). Interestingly, the sequencing of a number of mycobacteriophage genomes has revealed that phage-tail-associated tape measure proteins from mycobacteriophages contain a number of domains, some of which are similar to those involved in host-encoded peptidoglycan-degrading enzymes (Piuri & Hatfull, 2006), suggesting that these additional domains possibly aid viral DNA
injection via localized cell wall degradation, and thus play an important role in initiating infection. However, these early events in phage infection are dependent on, and preceded by, the specific binding of phage to its cell surface receptor on the bacterial envelope. In this study, we used transposon mutants and defined glycosyltransferase mutants to identify GPLs as the \textit{M. smegmatis} cell surface receptors for phage I3, which belongs to the family \textit{Myoviridae}.

Interestingly, \textit{Mycobacterium avium}, which also produces GPLs, seems naturally resistant to phage I3 (Lee \textit{et al.}, 2004). This may be either because a rough variant of \textit{M. avium} (that does not produce GPLs) was used to assay infectivity, or because the glycosylation of the deoxytalose residue in \textit{M. avium} GPLs may have a ‘masking’ effect that prevents the recognition of methylated rhamnose by phage I3. \textit{M. tuberculosis}, on the other hand, is naturally resistant to phage I3 because it does not synthesize GPLs.

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Simplified representations of the structures of GPLs found in \textit{M. smegmatis} mc\textsuperscript{2}155 (a), and biosynthetic intermediates that accumulate in \textit{Δgtf1} (b, c) and \textit{Δgtf2} (d) mutants. Rha, rhamnose; 6-\textit{d}-Tal, 6-deoxytalose; Me, methyl. Variations in the fatty acyl chain (R) and acetyl modifications of talose are not shown.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig4}
\caption{TLC analysis of polar lipids extracted from parental (mc\textsuperscript{2}155) and phage-I3-resistant mutants. (a–e) Autoradiographs of [\textsuperscript{14}C]-labelled polar lipids resolved using chloroform/methanol/water (100:14:0.8) for direction I, and chloroform/acetone/methanol/water (50:60:2.5:3) for direction II. (a) mc\textsuperscript{2}155, (b) I3R-1, (c) I3R-2, (d) I3R-3, (e) I3R-4. (f) Deacetylated polar lipids were also run using the solvent system chloroform/methanol/water (90:10:1) to visualize GPLs 1, 2, 3 and 4.}
\end{figure}
Apart for the identification of the phage I3 receptor, this work also highlighted the utility of phage-resistance screens to identify genes involved in the biosynthesis/transport of cell envelope components, as we isolated mutants defective in three different classes of genes (one glycosyltransferase, two non-ribosomal peptide synthases and one mbtH-like gene of unknown activity), all of which are involved in GPL biosynthesis. While in this case most of the genes involved in GPL biosynthesis have been studied in detail in the past, the availability of numerous mycobacteriophages would allow screening for mutants defective in other cell wall components that constitute the corresponding phage receptors. Indeed, this work showed that the phages D29 and Bxz1 have receptor(s) distinct from that for phage I3, as the I3-resistant mutants retained sensitivity to these phages. More than 250 mycobacteriophages have been isolated to date (Barksdale & Kim, 1977; Hatfull & Jacobs, 1994), and only a few have been studied in detail (Hatfull, 2005). Mycobacteriophages show diversity in genome sequences, morphology and host range (Pedulla et al., 2003; Sampson et al., 2009), and it is quite likely that this diversity is also reflected in the nature of their corresponding receptors in the cell walls of mycobacteria. In addition, studies on phage receptors may aid the further development of phage typing as a diagnostic tool for the identification of strains of pathogenic mycobacteria that produce variants of cell surface components.

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