Biosynthetic specificity of the rhamnosyltransferase gene of *Mycobacterium avium* serovar 2 as determined by allelic exchange mutagenesis

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In prior studies, through recombinant expression in *Mycobacterium smegmatis*, the *rtfA* gene of *Mycobacterium avium* was shown to encode a rhamnosyltransferase that catalyses the addition of rhamnose (Rha) to the 6-deoxytalose of serovar 2-specific glycopeptidolipid (GPL). Whether RtfA also catalyses the transfer of Rha to the alaninol of the lipopeptide core is unknown. An isogenic *rtfA* mutant of *M. avium* serovar 2 strain TMC724 was derived using a novel allelic exchange mutagenesis system utilizing a multicopy plasmid that contained the *katG* gene of *Mycobacterium bovis* and the gene encoding green fluorescent protein (*gfp*). Overexpression of KatG in *M. avium* resulted in increased susceptibility to isoniazid, thus providing counter-selection by enriching for clones that had lost plasmid DNA. Plasmid loss was confirmed by screening for *gfp*-negative clones to select putative allelic exchange mutants. Two exchange mutants were created, confirmed by Southern hybridization, and demonstrated loss of serovar 2-specific GPL by thin-layer chromatography (TLC). Gas chromatography of alditol acetate derivatives revealed the loss of Rha and the terminal 2,3-O-Me-fucose and preservation of 3-O-Me-Rha and 3,4-O-Me-Rha substituents at the terminal alaninol of the lipopeptide core. Complementation of *rtfA in trans* through an integrative plasmid restored serovar 2-specific GPL expression identical to wild-type TMC724. This result shows that *rtfA* encodes an enzyme responsible only for the transfer of Rha to the serovar 2-specific oligosaccharide and provides a system of allelic exchange for *M. avium* as a tool for future genetic studies involving this species.

INTRODUCTION

Members of the *Mycobacterium avium* complex (MAC) are ubiquitous organisms found in soil and natural bodies of water and are common colonizers of potable hot water systems (Brooks *et al.*, 1984; George *et al.*, 1980; von Reyn *et al.*, 1993, 1994). Although initially considered as an opportunistic pulmonary pathogen, disseminated MAC infection, especially among patients with end-stage AIDS, was described in many reports in the 1980s and 1990s (Hawkins *et al.*, 1986; Horsburgh, 1991; Nightingale *et al.*, 1992). In the last 10 years, there have been significant advances in antiretroviral treatment of HIV infection as well as the introduction of prophylactic regimens to prevent the onset of disseminated MAC (DMAC) infection (Havlir *et al.*, 1996; Pierce *et al.*, 1996; Shafran *et al.*, 1996). Although the incidence of DMAC infection has been significantly reduced, this disease continues to cause significant morbidity among patients with AIDS (Burman *et al.*, 1998).

The pathogenesis of DMAC is largely unresolved. Numerous bacterial molecules have been postulated as candidate virulence factors or factors that modulate the host cellular response. The glycopeptidolipids (GPLs), the dominant MAC glycolipid (Brennan, 1988; McNeil *et al.*, 1987), have long been considered to have a role in pathogenesis (Draper & Rees, 1970, 1973); however, there are conflicting data as to the relative effect of GPL from different serovars and the relative contribution of each GPL subunit. Heat-killed staphylococci coated with serovar 4, 12 and 17 GPL strongly promoted macrophage uptake and markedly inhibited phagosome–lysosome (PL) fusion, GPL of serovars 5, 7, 8

Abbreviations: 6-dTal, 6-deoxytalose; GPL, glycopeptidolipid; INH, isoniazid; LP, lipopeptide; nsGPL, non-specific glycopeptidolipid; Rha, rhamnose; ssGPL, serovar-specific glycopeptidolipid.
and 10 stimulated both phagocytosis and PL fusion, and GPL of serovars 9, 13, 16 and 19 neither promoted phagocytosis nor inhibited PL fusion (Minami, 1998). Using whole bacteria, a virulent serovar 4 strain of \textit{M. avium} induced protein kinase C-dependent TNFα, IL-1β and IL-6 (Gan \textit{et al}., 1993; Newman \textit{et al}., 1991), effects that appear to be related to GPL alone (Barrow \textit{et al}., 1995). In contrast, others have reported that serovar 8-specific GPL induced significant levels of TNFα, IL-1β and IL-6 from human peripheral blood mononuclear cells (PBMCs) and serovar 4 GPL did not (Barrow \textit{et al}., 1995; Horgan \textit{et al}., 2000). While two groups of investigators demonstrated that, following β-elimination of GPL, the modified lipopeptide (LP) core of serovar 4-specific GPL, as opposed to the reduced oligosaccharide, inhibited mitogen-induced blastogenesis (Tassell \textit{et al}., 1992) and suppressed phytohaemagglutinin-induced lymphoproliferation of PBMCs (Barrow \textit{et al}., 1995), others have reported that the LP core of serovar 4-specific GPL had only a minimal ability to induce TNFα relative to unmodified GPL (Barrow \textit{et al}., 1995). Significantly limiting the development of a consistent framework of the role of GPL in mycobacterial pathogenesis has been the inability to construct isogenic strains differing in GPL structure, necessitating the use of genetically distinct strains of differing serotypes.

Two forms of GPL are expressed on smooth morphotypes of \textit{M. avium}: serovar-specific glycopeptidolipid (ssGPL) and non-specific glycopeptidolipid (nsGPL). Both nsGPs and ssGPs possess a LP core composed of d-phenylalanine-d-allo threonine-d-alanine-L-alaninol (Phe-Thr-Ala-alaninol) with a fatty acid N-linked to Phe, a 6-deoxyltalose (6-dTal) substitution on the allo Thr, and mono- or dimethylated rhamnose (Rha) linked to the C terminus of alaninol. The nsGPs are further glycosylated with a variable haptenic oligosaccharide (Aspinnall \textit{et al}., 1995). For all serovars of the \textit{M. avium} complex, except serovars 5, 10 and 11, Rha appears as the first sugar moiety attached to the 6-dTal residue (Aspinnall \textit{et al}., 1995) and was therefore chosen as the initial target for study within the serovar gene cluster.

Genes encoding the glycosylation of the serovar 2-specific GPL are localized to a contiguous 35 kb region on the \textit{M. avium} chromosome termed the ser2 gene cluster (Belisle \textit{et al}., 1991). Four loci, serA–D, were identified and demonstrated to encode putative glycosyltransferases, methyltransferases and genes for the \textit{de novo} synthesis of l-fucose (Belisle \textit{et al}., 1993a, b; Mills \textit{et al}., 1994). Biosynthesis of the LP core was shown to reside downstream of the ser2 gene cluster (Belisle \textit{et al}., 1993b). Within the ser2A locus, three open reading frames (ORFs 1–3) were identified, including two putative glycosyltransferases and a methyltransferase (Eckstein \textit{et al}., 1998). Recombinant expression in \textit{Mycobacterium smegmatis} ORF2 encodes a glycosyltransferase that links Rha to 6-dTal, and was termed rtfA (Eckstein \textit{et al}., 1998). However, these experimental data could not rule out that RtfA was also responsible for the transfer of Rha to the L-alaninol of the GPL LP core.

To demonstrate clearly the role of rtfA in the biosynthesis of ssGPL, an rtfA deletion mutant was created in serovar 2 strain TMC724 and represents the first successful creation of allelic exchange mutants of \textit{M. avium}. This was accomplished using an episomal vector carrying the katG gene of \textit{M. bovis} BCG to confer isoniazid (INH) susceptibility and provided the ability to counter-select for clones that had lost plasmid DNA. Positive co-selection was accomplished with the hygromycin-resistance gene (hyg) and the green fluorescent protein (gfp) gene. Complementation of the rtfA knockout mutant with wild-type rtfA restored GPL expression. Thus, we were able to demonstrate that disruption of rtfA resulted in the inability to glycosylate 6-dTal but did not disrupt the transfer of Rha to the L-alaninol of the LP core.

**METHODS**

**Bacterial strains and chemicals.** The mycobacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. \textit{Escherichia coli} DH5α was used as the host strain for plasmid construction and propagation. Growth and selection of recombinant \textit{E. coli} clones were carried out using Luria–Bertani (LB) medium (American Bioanalytical) with 50 μg ampicillin ml⁻¹ or 25 μg kanamycin ml⁻¹. \textit{Mycobacterium} spp. were grown in Middlebrook 7H9 broth or 7H11 agar (Difco) supplemented with 10% OADC (oleic acid, albumin, dextrose complex). \textit{M. smegmatis} mc²155 is a transformation competent strain that is widely used to express or carry recombinant mycobacterial genes (Snapper \textit{et al}., 1990). \textit{M. avium} TMC724 is a serovar 2 strain for which the region encoding GPL biosynthesis has been isolated and sequenced (GenBank accession no. AF125999) (Belisle \textit{et al}., 1991; Eckstein \textit{et al}., 1998; Mills \textit{et al}., 1994). \textit{M. avium} 920A-6 is an electrocompetent, serovar 8 bloodstream isolate cultured from a patient with AIDS (Arbeit \textit{et al}., 1993; Lee \textit{et al}., 2002). For \textit{M. smegmatis} and \textit{M. avium}, selection of recombinant clones was accomplished using hygromycin at 100 μg ml⁻¹ or kanamycin at 50 μg ml⁻¹. All chemicals were purchased from Sigma.

**Bacterial transformation.** Transformation of \textit{E. coli} was performed using calcium-chloride-treated cells (Sambrook \textit{et al}., 1989). Transformation of \textit{M. smegmatis} was performed by electroporation as described by Snapper \textit{et al}., 1990). Transformation of \textit{M. avium} was performed by electroporation as described by Lee \textit{et al}., 2002).

**Isolation and identification of the BCG katG gene.** The \textit{katG} gene was isolated from an \textit{M. bovis} BCG genomic library contained in the mycobacterium–\textit{E. coli} shuttle vector pYUB415 (kindly provided by Stoyan Bardarov). This genomic library was used to transform \textit{M. smegmatis} mc²155 and five putative \textit{katG}-positive transformants were identified as susceptible to INH at 1 μg ml⁻¹. The presence of \textit{katG} in each of the five transformants was confirmed by PCR. Bacterial lysates for PCR analyses were derived from late-exponential-phase cultures grown on selective agar. Bacteria were suspended in PBS (40 mM KH₂PO₄, 60 mM K₂HPO₄, pH 7.0) and heated to 95 °C for 10 min. After centrifugation at 15 000 g, the supernatant was recovered as a DNA lysate. The forward primer 5'-GAGATTGCCAGCCTAAGAGC-3' corresponded to base positions 1360–1380 of the \textit{Mycobacterium tuberculosis} katG gene (GenBank accession no. X68081; Zhang \textit{et al}., 1992) and the reverse primer 5'-CAGGTGATACCCATGTGCAG-3' corresponded to base
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>M. smegmatis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mc²155</td>
<td>Transformable competent isolate of mc²6</td>
<td>Snapper et al. (1990)</td>
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<tr>
<td>127R</td>
<td>mc²155(pVAP1); katG hyg oriM</td>
<td>This study</td>
</tr>
<tr>
<td>146R.1</td>
<td>mc²155(pVAP9.2); katG hyg L5 attP–int</td>
<td>This study</td>
</tr>
<tr>
<td>173R.1</td>
<td>mc²155(pVAP25); katG hyg</td>
<td>This study</td>
</tr>
<tr>
<td><strong>M. avium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>920A-6</td>
<td>Serovar 8 clinical strain, electrocompetent</td>
<td>Arbeit et al. (1993); Lee et al. (2002)</td>
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<tr>
<td>174R</td>
<td>920A-6(pVAP1); katG hyg oriM</td>
<td>This study</td>
</tr>
<tr>
<td>185R</td>
<td>920A-6(pVAP9.2); katG hyg L5 attP–int</td>
<td>This study</td>
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<tr>
<td>186R</td>
<td>920A-6(pVAP25); katG hyg</td>
<td>This study</td>
</tr>
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<td>TMC724</td>
<td>Serovar 2 strain</td>
<td>ATCC</td>
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<td>218R.4</td>
<td>TMC724; rtfA::hyg</td>
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<td>235R.1</td>
<td>218R.4(pVAP52); rtfA</td>
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Table 2. Plasmids used in this study

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<th>Plasmid</th>
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<tr>
<td>pBluescript SK (+/-)</td>
<td>oriE bla lacZ</td>
<td>Invitrogen</td>
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<td>pMVGF2</td>
<td>pAL5000 oriM, pUC19 oriE kan gfp</td>
<td>Lee et al. (2002)</td>
</tr>
<tr>
<td>pIGFP2</td>
<td>L5 att-int, pUC19 oriE kan gfp</td>
<td>Lee et al. (2002)</td>
</tr>
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<td>pTRB21</td>
<td>pYUB18, ser2 region of M. avium TMC724</td>
<td>Belisle et al. (1991)</td>
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<tr>
<td>pNVB1</td>
<td>pAL5000 oriM, pUC19 oriE kan</td>
<td>Howard et al. (1995)</td>
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<td>pVAP1</td>
<td>M. bovis BCG katG in pYUB415</td>
<td>This study</td>
</tr>
<tr>
<td>pVAP9.2</td>
<td>40 kb PstI insert of pVAP1 in pYUB412, katG</td>
<td>This study</td>
</tr>
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<td>pVAP25</td>
<td>6 kb EcoRV fragment of pVAP1 in pNVB1, katG</td>
<td>This study</td>
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<td>pVAP36</td>
<td>1·9 kb rtfA amplicon in pBluescript SK (+/-)</td>
<td>This study</td>
</tr>
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<td>pVAP37</td>
<td>1·3 kb BamHI hyg cassette in BglII site of rtfA in pVAP36</td>
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<td>pVAP40</td>
<td>3·2 kb PstI–HindIII rtfA::hyg from pVAP37 in pMVGF2</td>
<td>This study</td>
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<td>pVAP41</td>
<td>katG in 6 kb EcoRV fragment of pVAP1 in pVAP40</td>
<td>This study</td>
</tr>
<tr>
<td>pVAP52</td>
<td>pIGFP2 containing the 1·9 kb rtfA amplicon</td>
<td>This study</td>
</tr>
<tr>
<td>pYUB412</td>
<td>L5 att-int, pUC19 oriE hyg bla,Padr MCS*</td>
<td>Lee et al. (2002)</td>
</tr>
<tr>
<td>pYUB415</td>
<td>pAL5000 oriM, pUC19 oriE hyg bla, Padr MCS*</td>
<td>This study</td>
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</table>

*The multi-cloning site (MCS) of pYUB412 and pYUB415 was derived from pUC19 and engineered with flanking PstI restriction sites (S. Bardarov, personal communication).

Integrating cosmid pVAP9.2 was constructed by ligating the 40 kb PstI insert of pVAP1 to PstI/XbaI arms of pYUB412. The ligation product was packaged into E. coli DH5α and retransformed into M. smegmatis to confirm INH susceptibility.

The stability of the pAL5000-based vector pVAP41 was determined for M. avium strain 920A-6. Following transformation with

in Fig. 1. The rtfA gene was amplified from pTRB21 (Belisle et al., 1991). The forward primer 5’-GCCCAGATCTGAGGAGCATT-3’ (SHL01) and reverse primer 5’-GCTGCCATACTGTCAGATC-3’ (SHL02) corresponded to positions 4030–4051 and 5924–5944, respectively, of the ser2 gene cluster of TMC724 (GenBank accession no. AF125999). PCR was performed using a programme of 35 cycles of 94°C for 1 min, 62°C for 2·5 min and 72°C for 1 min. The 1914 bp PCR product was ligated into the EcoRV site of pBluescript SK (+/-) to create pVAP36 (4·8 kb). The 1·3 kb BamHI hyg cassette from pBSGHG (Lee et al., 2002) was inserted via blunt-end ligation into the unique Ncol site within rtfA to create pVAP37 (6·2 kb). The 3·2 kb HindIII–PstI fragment of pVAP37 containing rtfA::hyg was ligated into the respective sites in the multiple cloning region of pMVGF2 (Lee et al., 2002) to create pVAP40 (8·3 kb). Finally, katG, contained within the 6 kb EcoRV fragment of pVAP1, was ligated into the Nhel site of pVAP40 to create pVAP41 (14·1 kb).
pVAP41, a gfp-positive, hygromycin-resistant clone was propagated non-selectively in 7H9 broth. Serial dilutions were inoculated onto selective 7H11 agar containing 100 µg hygromycin ml⁻¹. After 13 days of non-selective incubation, 24-3% of colonies remained hygromycin-resistant versus only 5-8% of colonies after 20 days incubation. All colonies were green fluorescent protein (GFP)-positive, demonstrating

Fig. 1. Construction of allelic exchange vector pVAP41. The rtfA gene was amplified as a 1914 bp product from pJTB21 (Belisle et al., 1991) using forward primer SHL01 and reverse primer SHL02 and ligated into the EcoRV site of pBluescript SK (+/-) to create pVAP36 (4-8 kb). The 1-3 kb hyg BamHI cassette of pBSGHG (Lee et al., 2002) was inserted via blunt-end ligation into the unique NcoI site within rtfA to create pVAP37 (6-2 kb). The 3-2 kb HindIII-PstI fragment of pVAP37 containing rtfA::hyg was ligated into the respective sites in the multiple cloning region of pMVGFP2 (Lee et al., 2002) to create pVAP40 (8-3 kb). Finally, the 6 kb EcoRV fragment of pVAP1 containing karG was ligated into the Nhel site of pVAP40 to create pVAP41 (14-1 kb).
a very low rate of spontaneous hygromycin resistance. Based on these data, the half-life of pVAP41 for M. avium appears to range between 5 and 6-5 days. These data were consistent for two separate cultures.

Plasmid pVAP52 was constructed as an integrative vector to complement the interrupted rtfA gene. To construct plasmid pVAP52, rtfA was amplified using primers SHL01 and SHL02, except that SHL01 was extended upstream by 12 bp (5'-ACCATAAGCTT-3') to include a HinIII restriction site (underlined) and SHL02 was extended downstream by 11 bp (5'-ACTCCAAGCTT-3') to include a HindIII restriction site. After restriction digestion with HindIII, this fragment was ligated into the HindIII site of pGFP2 (Lee et al., 2002).

**Isolation of genomic DNA.** Genomic DNA for Southern blot analysis was isolated from 30 ml late-exponential-phase cultures. Bacteria were washed with 30 ml TES (100 mM Tris/HCl, pH 8-0, 100 mM EDTA, pH 8-0, 100 mM NaCl), suspended in 2-5 ml of TES with 300 µl lysosome (10 mg ml⁻¹), and incubated at 37 °C for 1-5 h. Proteinase K and SDS were added to final concentrations of 1 mg ml⁻¹ and 1% (w/v), respectively, followed by incubation at 55 °C for 5 h. Phenol/chloroform/isooamy alcohol (25:24:1 by vol.) was added at 1-5 vol., the suspension was vortexed for 2 min and then centrifuged at 15 000 g for 15 min at room temperature. The aqueous layer was transferred into a new microcentrifuge tube and 0-7 vol. of 2-propanol was added. After incubation at room temperature for 5 min, genomic DNA was collected by centrifugation at 15 000 g for 10 min at room temperature and washed with 70% ETOH. The DNA pellet was air-dried and suspended in H₂O.

Southern blot analysis was performed for genomic DNA subjected to overnight restriction digestion with HindIII (Promega) and resolved in a 0-8% agarose gel at 35 V for 18 h. The restriction fragments were transferred to a non-charged nylon membrane (Duralon-UV; Schleicher and Schuell) by capillary action (Sambrook et al., 1989). Southern blots were probed for rtfA using a 1-9 kb PCR amplicon generated as above using the NorthSouth Direct HRP labelling and detection kit (Pierce), following the manufacturer’s directions.

**Isolation and analysis of GPLs.** GPLs were isolated from lyophilized 100 ml bacterial cultures. Total lipids were extracted with CHCl₃/CH₃OH (2:1, v/v) and enrichment for GPLs was achieved by mild alkaline hydrolysis as described previously (Eckstein et al., 1998). The GPLs (100 µg) were resolved by TLC on 20 cm x 20 cm silica gel 60 plates (Merck) using CHCl₃/CH₃OH/H₂O (90:10:1 by vol.) to include a HindIII site (underlined) and SHL02 was extended downstream by 11 bp (5'-ACTCCAAGCTT-3') to include a HindIII restriction site. After restriction digestion with HindIII, this fragment was ligated into the HindIII site of pGFP2 (Lee et al., 2002).

Carbohydrate analysis of the GPLs from the wild-type and recombinant strains indicated increased susceptibility to INH compared to wild-type M. smegmatis. All transformants demonstrated increased susceptibility to INH compared to wild-type M. smegmatis. Strains 127R and 146R, carrying large episcopal and integrative cosmids, demonstrated a 4–5 log decrease in cell numbers at an INH concentration of 0.25 µg ml⁻¹ and were fully susceptible at 0.5 µg ml⁻¹, whereas strain 173R1 was fully susceptible to INH at a concentration of 0.05 µg ml⁻¹. KatG-mediated INH susceptibility was further tested for recombinant clones 174R, 185R and 186R of M. avium 920A-6 SmO (Lee et al., 2002) carrying plasmids pVAP1, pVAP9.2 and pVAP25, respectively. Clones 174R and 185R demonstrated a pattern of INH susceptibility similar to wild-type 920A-6 (Fig. 2a) whereas clone 186R was highly susceptible to INH and demonstrated a 3-5 log decrease in cell numbers at an INH concentration of 0.5 µg ml⁻¹ (Fig. 2a).

**RESULTS**

**M. avium** cells transformed with *katG* display increased susceptibility to INH

The rtfA gene of M. avium strain TMC724 was chosen as our initial target to examine the biosynthesis of GPL. Preliminary experiments first investigated whether an allelic exchange system based on pPR27 (Pellic et al., 1996a), containing both sacB and the temperature-sensitive origin of replication for Mycobacterium fortuitum plasmid pAL5000, could be used for M. avium. Electromagnetic M. avium strain 920A-6 was transformed with a pPR27-based plasmid, pVAP39 (data not shown). Both sucrose and temperature selection provided only minimal decreases in cell counts (<50%; data not shown). Moreover, transformation of TMC724, a strain with a baseline low efficiency of transformation (Lee et al., 2002), could not be successfully electroporated with pVAP39. Thus, to generate such mutants in M. avium a more amenable counter-selection strategy was required.

KatG-mediated INH selection has been used successfully as a counter-selection marker for allelic exchange of M. bovis BCG (Norman et al., 1995). Given that M. avium is naturally resistant to relatively high concentrations of INH, we sought to determine whether an episomal copy of the M. bovis BCG KatG gene could confer INH susceptibility to M. avium and be used as a marker to select mutants derived by homologous recombination.

Initially, M. smegmatis was transformed with the *katG*-containing plasmids pVAP1, pVAP9.2 and pVAP25 to create strains 127R, 146R1 and 173R1, respectively. INH susceptibility of each M. smegmatis transformant was compared to wild-type M. smegmatis. All transformants demonstrated increased susceptibility to INH compared with wild-type M. smegmatis. Strains 127R and 146R, carrying large episcopal and integrative cosmids, demonstrated a 4–5 log decrease in cell numbers at an INH concentration of 0.25 µg ml⁻¹ and were fully susceptible at 0.5 µg ml⁻¹, whereas strain 173R,1 was fully susceptible to INH at a concentration of 0.05 µg ml⁻¹. KatG-mediated INH susceptibility was further tested for recombinant clones 174R, 185R and 186R of M. avium 920A-6 SmO (Lee et al., 2002) carrying plasmids pVAP1, pVAP9.2 and pVAP25, respectively. Clones 174R and 185R demonstrated a pattern of INH susceptibility similar to wild-type 920A-6 (Fig. 2a) whereas clone 186R was highly susceptible to INH and demonstrated a 3-5 log decrease in cell numbers at an INH concentration of 0.5 µg ml⁻¹ (Fig. 2a).

Finally, KatG-mediated INH susceptibility was assessed for the episomal delivery vector pVAP41. Strain 920A-6 SmO transformed with pVAP41 demonstrated bacterial killing intermediate between pVAP25 and pVAP1 with a 6 log decline in bacterial numbers at 0.5 µg ml⁻¹ but no further increases in INH susceptibility (data not shown). Wild-type M. avium strain TMC724 was fully resistant to INH ≤10 µg ml⁻¹ and demonstrated an ~4.5 log decline at INH concentrations ≥25 µg ml⁻¹, whereas TMC724 transformed with pVAP41 demonstrated a 6-2 log decline at 10 µg ml⁻¹ (Fig. 2b). These data further demonstrated that KatG-mediated INH susceptibility could be used to select mutants that had undergone homologous recombination.
but that the level of KatG-mediated INH susceptibility and optimal concentration was highly strain-dependent.

**Allelic exchange mutagenesis of *rtfA* of serovar 2 strain TMC724**

The episomal delivery vector (pVAP41) was constructed to contain *rtfA* interrupted with the hygromycin-resistance gene (*rtfA::hyg*), KatG and gfp. TMC724(pVAP41) transformants were grown non-selectively to allow for homologous recombination and then inoculated onto INH- and hygromycin-containing medium. INH-susceptible clones that retained episomal copies of plasmid DNA should be eliminated, whereas clones that had lost plasmid DNA would be retained. Conversely, hygromycin selection would select for INH-resistant clones that had undergone either homologous recombination with *rtfA::hyg* for *rtfA* or chromosomal recombination of plasmid DNA via single crossover mutagenesis or by illegitimate recombination. The loss of gfp-mediated fluorescence (Lee et al., 2002) could then be used to detect clones that had undergone homologous recombination.

*M. avium* strain TMC724 electroporated with 1 µg of pVAP41 yielded four GFP-positive colonies. Each of the four clones yielded both a 1·9 kb PCR product corresponding to wild-type *rtfA* and a 3·2 kb product corresponding to *rtfA* interrupted with *hyg* (*rtfA::hyg*). One transformant was grown non-selectively in 7H9 medium for 24 days to provide sufficient time for homologous recombination and to allow for spontaneous plasmid loss. The cell suspension was then inoculated onto 7H11 medium supplemented with INH and hygromycin to select for INH-resistant clones that had undergone either homologous recombination with *rtfA::hyg* for *rtfA* or chromosomal recombination of plasmid DNA via single crossover mutagenesis or by illegitimate recombination. The loss of gfp-mediated fluorescence (Lee et al., 2002) could then be used to detect clones that had undergone homologous recombination.

PCR for *rtfA* was then performed for the six clones. Three clones (218R.3, 218R.4 and 218R.5) yielded a single 3·2 kb PCR product consistent with *rtfA::hyg*. Clone 218R.6 yielded both a 1·9 kb PCR product and a 3·2 kb band, signifying the presence of wild-type *rtfA* and *rtfA::hyg*. Clones 218R.1 and 218R.2 yielded no detectable PCR products, representing probable spontaneous deletion mutants in the genes encoding GPL biosynthesis (Belisle et al., 1993b).

Southern blot analysis was performed to confirm the genotype of the allelic exchange mutants (Fig. 3). Genomic DNA from strain 218R.4 was digested with HindIII (there

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**Fig. 2.** INH susceptibility of wild-type and katG-transformants of *M. avium*. (a) INH susceptibility of *M. avium* 920A-6 (●) and 920A-6 katG transformants: 174R [920A-6(pVAP1); □], 185R [920A-6(pVAP9.2); ▲] and 186R [920A-6(pVAP25); ◆]. (b) INH susceptibility of TMC724 (●) and TMC724(pVAP41) (◆). Plasmid pVAP1 contains katG as part of a 48 kb episomal cosmid; plasmid pVAP9.2 contains katG as part of a 48 kb integrative cosmid; plasmid pVAP25 contains katG as part of an 11·8 kb episomal plasmid and plasmid pVAP41 contains katG as part of a 14·1 kb episomal plasmid.

**Fig. 3.** Southern blot analysis of allelic exchange mutants. Genomic DNA from wild-type TMC724 and clone 218R.4 was digested with HindIII (there

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19 kb

18 kb

17 kb
are no *Hin*dIII sites in either *rtfA* or *hyg*) and hybridized for *rtfA*. Wild-type TMC724 yielded a 17-4 kb restriction fragment that hybridized to *rtfA*. In comparison, clone 218R.4 yielded an 18-7 kb hybridizing fragment corresponding to a 1-3 kb increase from the *hyg* insert.

**RtfA encodes addition of Rha to 6-dTal and not to the alaninol of the LP core as confirmed by TLC and GC-MS**

It was hypothesized that the creation of an insertional deletion mutant in *rtfA* of TMC724 via allelic exchange would prevent the addition of Rha as the proximal serovarspecific carbohydrate to 6-dTal within the GPL core (Eckstein *et al*., 1998). The *rtfA* knockout mutants should thus express only the nsGPL and be devoid of Rha and 2,3-O-Me-fucose (2,3-O-Fuc). As expected, TLC of alkaline stable lipids (Fig. 4) demonstrated that TMC724 expressed both ssGPL and nsGPL, whereas clones 218R.4 and 218R.5 expressed only nsGPL. For both mutant clones, the pattern of nsGPL expression was unchanged compared to the wild-type serovar 2 strain (Fig. 4, and data not shown).

CG analysis of the alditol acetate derivatives yielded five peaks for TMC724 corresponding to 6-dTal, Rha, 2,3-O-Me-Fuc, 3-O-Me-Rha and 3,4-O-Me-Rha (Fig. 5). The spectrum of sugars from clone 218R.5 showed loss of the Rha peak and the distal 2,3-O-Me-Fuc and preservation of 3-O-Me-Rha and 3,4-O-Me-Rha. Relative to wild-type bacteria, however, the *rtfA* mutant displayed an increase in 3-O-Me-Rha. This confirms the conclusion of Eckstein *et al*. (1998) that *rtfA* is responsible for the addition of Rha to 6-dTal as
the proximal sugar of serovar 2-specific GPL, but does not catalyse the addition of Rha to alaninol of the LP core.

Finally, proof that disruption of \( rtfA \) resulted in loss of serovar 2-specific GPL expression was confirmed by complementation of \( rtfA \) in \( \text{trans} \). Clone 218R.4 was transformed with the integrative plasmid pVAP52 containing wild-type \( rtfA \). TLC of this clone (235R.1) showed recovery of the baseline pattern for GPL expression (Fig. 4).

**DISCUSSION**

Site-directed mutagenesis via allelic replacement had been described for *Mycobacterium intracellulare* (Marklund et al., 1995), *M. bovis* BCG (Aldovini et al., 1993; Norman et al., 1995; Pelicic et al., 1996a; Wards et al., 2000), *M. tuberculosis* (Pelicic et al., 1997) and *M. smegmatis* (Husson et al., 1990), and has been extended to include *M. avium* in this report. By *in situ* replacement of \( rtfA \) with \( rtfA::hyg \), we were able to confirm that RtfA catalyses the addition of Rha only to 6-dTal as the first serovar-specific sugar but does not catalyse the addition of Rha to alaninol of the LP core of GPL. Thus, through the construction of mutants in GPL biosynthesis, to include \( rtfA \), the role of GPL in the pathogenesis of *M. avium* can be studied in a consistent manner.

Homologous recombination techniques for mycobacteria have typically utilized replicative plasmids (Baulard et al., 1996) to allow for greater time for single crossover versus use of linear strands of non-replicative DNA (Balasubramanian et al., 1996). In fact, for *M. intracellulare*, homologous recombination has been shown to require an intermediate state created by a single crossover with subsequent elimination of plasmid DNA sequences by a double crossover state. Enrichment of allelic exchange mutants that have lost plasmid DNA is facilitated by the inclusion of selection and counter-selection markers allowed as part of the vectors (Pelicic et al., 1997).

Counter-selection markers used for the successful creation of allelic exchange mutants of *M. tuberculosis* and *M. smegmatis* employed a plasmid vector that incorporated a temperature-sensitive origin of replication (ts-OriM) and contained the \( sacB \) gene (Pelicic et al., 1996a, b, 1997). Unfortunately, we found that neither \( sacB \) nor the ts-OriM provided minimal if any selection for *M. avium*. Moreover, the lowered incubation temperatures required for the ts-OriM (Lee et al., 2002) precluded successful transformation strains with lower transformation efficiencies such as TMC724. In addition, while other genes such as \( rpsL \) have been used for counter-selection for mycobacteria (Sander et al., 1995), their use for *M. avium* also appears limited (D. Carroll, personal communication). Thus, other selection and counter-selection markers were investigated.

Exploitation of INH susceptibility, conferred by introduction of \( katG \) from a susceptible species of mycobacteria, as well as inclusion of \( gfp \), allowed for a facile method to select for double exchange mutants that had undergone loss of plasmid DNA. The use of KatG as a counter-selection marker for *M. avium* was, however, shown to be strain-specific and required careful study to determine the optimal concentration of INH. We showed that, for *M. avium* strain TMC724, an INH concentration of 10 \( \mu \text{g ml}^{-1} \) would provide an approximate 1 log decrease in cell numbers between wild-type bacteria and strains transformed with \( katG \) contained on an episomal plasmid, whereas for *M. avium* strain 920A-6, INH at a concentration of 1 \( \mu \text{g ml}^{-1} \) yielded an 8 log decrease in cell numbers for transformed bacteria. Similar results were observed for the intrinsically more resistant smooth transparent morphotype of strain 920A-6. Our data would predict that once chromosomal integration of plasmid DNA occurred via an initial single crossover event [such as occurs for *M. intracellulare* (Marklund et al., 1997)], there would be no additional selection with KatG-mediated INH selection. Of note is that our data for serovar 8 strain 920A-6 corroborate studies that INH resistance of *M. avium* does not appear to result from a diffusion barrier across the cell wall (Mdluli et al., 1998). Unlike *M. smegmatis*, a single copy of \( katG \) did not confer INH susceptibility to *M. avium*, suggesting possible interference from the presence of the native *M. avium* \( katG \) gene.

The genetic basis of the biosynthesis of the *M. avium* serovar 2-specific GPL has been the subject of prior studies (Belisle et al., 1991; Eckstein et al., 1998; Mills et al., 1994). Oligosaccharide biosynthesis was initially localized to a 28 kb chromosomal region of TMC724 (Belisle et al., 1991) that was demonstrated to contain four functional domains, \( ser2A \) to \( ser2D \) (Mills et al., 1994). Transposon mutagenesis showed that the genes encoding the addition of serovar-specific oligosaccharides resided within \( ser2A \), the region associated with rhamnosyltransferase activity being contained within a 4.5 kb region within this locus (Mills et al., 1994). Sequence analysis of \( ser2A \) demonstrated three ORFs with homology to mycobacterial glycosyltransferases (ORF1 and ORF2) and a putative methyltransferase (ORF3) (Eckstein et al., 1998). Recombinant expression of ORF1 and ORF2 in *M. smegmatis* demonstrated that ORF2 was able to catalyse the addition of Rha to 6-dTal of the LP core of GPL, consistent with ORF2 representing the *M. avium* \( rtfA \) gene.

While prior work (Eckstein et al., 1998) was able to demonstrate that ORF2 could catalyse the addition of Rha to the 6-dTal of the LP core, proof of this function in *M. avium* was yet to be proven. Through the construction of site-directed knockout in the *M. avium* \( rtfA \) gene and subsequent complementation with the wild-type gene, we were able to conclusively prove that RtfA catalyses the addition of Rha as the proximal sugar in the serovar-specific oligosaccharide. GC analysis of alditol acetate derivatives of GPL in the prior study (Eckstein et al., 1998) showed an increase in 2,3,4-O-Me-Rha relative for the \( rtfA \) transformant to wild-type *M. smegmatis*. This result suggested the unlikely
posibility that rtfA could be bifunctional, catalysing both the addition of Rha to 6-dTal and the addition of Rha to alanolin of the LP core. We did not observe the same inversion of molar ratios of 6-dTal and 3,4-O-Me-Rha (2,3,4-O-Me-Rha in the prior study) showing that, as expected, RtfA catalyses only the addition of Rha to 6-dTal.

Using the techniques derived in this study, it is possible to consistently study the genetics of the biosynthesis of GPL and its role in the pathogenesis of *M. avium*. Disruption of rtfA represents an ideal first target in studies of pathogenesis since Rha represents the proximal sugar of the oligosaccharide moiety of ssGPL, independent of serovar. In this way, a major limitation of prior studies that have relied on comparisons of strains representing different serovars (Newman *et al*., 1991) or have chemically modified GPL to examine GPL subfractions (Barrow, 1991; Barrow *et al*., 1993; Brownback & Barrow, 1988; Tassell *et al*., 1992) can be avoided. Further, unresolved questions as to the function of other genes within the ser2A locus can be elucidated.

In summary, by site-directed mutagenesis, allelic exchange mutants were created in rtfA of serovar 2 strain TMC724. We were able to demonstrate conclusively that RtfA only catalyses transfer of Rha to 6-dTal of the LP core as the proximal serovar-specific sugar. Through the construction of this and additional mutants in GPL synthesis, the role of GPL in the host–pathogen interaction, immunogenesis and antibiotic resistance can be determined.

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