Deletion of the *mmpL4b* gene in the *Mycobacterium abscessus* glycopeptidolipid biosynthetic pathway results in loss of surface colonization capability, but enhanced ability to replicate in human macrophages and stimulate their innate immune response

Rachid Nessar,¹ Jean-Marc Reyrat,^{1†} Lisa B. Davidson²
and Thomas F. Byrd^{2,3}

Correspondence

Thomas F. Byrd
tbyrd@salud.unm.edu

¹Inserm-U 1002, Groupe Avenir, Université Paris V-Descartes, Faculté de Médecine, Site Necker, F-75730 Paris Cedex 15, France

²Department of Medicine, New Mexico Veterans Health Care System, 1501 San Pedro SE, Albuquerque, NM 87108, USA

³The University of New Mexico School of Medicine, Albuquerque, NM 87108, USA

Mycobacterium abscessus is considered to be the most virulent of the rapidly growing mycobacteria. Generation of bacterial gene knockout mutants has been a useful tool for studying factors that contribute to virulence of pathogenic bacteria. Until recently, the optimal genetic approach to generation of *M. abscessus* gene knockout mutants was not clear. Based on the recent identification of genetic recombineering as the preferred approach, a *M. abscessus* mutant was generated in which the gene *mmpL4b*, critical to glycopeptidolipid synthesis, was deleted. Compared to the previously well-characterized parental strain 390S, the *mmpL4B* deletion mutant had lost sliding motility and the ability to form biofilm, but acquired the ability to replicate in human macrophages and stimulate macrophage Toll-like receptor 2. This study demonstrates that deletion of a gene associated with expression of a cell-wall lipid can result in acquisition of an immunostimulatory, invasive bacterial phenotype and has important implications for the study of *M. abscessus* pathogenesis at the cellular level.

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INTRODUCTION

Mycobacterium abscessus is a rapidly growing non-tuberculous mycobacterium (NTM) which causes skin and soft tissue infections, and is an important cause of pneumonia in patients with structural abnormalities of the lung (De Groote & Huitt, 2006; Howard & Byrd, 2000; Griffith *et al.*, 1993). It is also a pulmonary pathogen in patients with cystic fibrosis (Cullen *et al.*, 2000; Fauroux *et al.*, 1997; Olivier *et al.*, 2003; Sermet-Gaudelus *et al.*, 2003). In some instances, isolation of *M. abscessus* from the sputum is not

associated with progressive lung infection and may be the result of transient airway colonization (Griffith *et al.*, 2007; Jönsson *et al.*, 2007). In other clinical situations, lung infection is progressive and refractory to antibiotic therapy, necessitating lung resection for cure (Griffith *et al.*, 1993; Daley & Griffith, 2002; Sherwood *et al.*, 2005). This latter clinical scenario has resulted in the view that *M. abscessus* is the most virulent of the rapidly growing mycobacteria that cause invasive lung infection (Petrini, 2006).

One of our laboratories has focused their studies on three well-characterized isogenic *M. abscessus* variants; 390R (rough colony morphology), 390S (smooth colony morphology) and 390V (rough colony morphology). *M. abscessus* 390R, a patient isolate, spontaneously dissociated to the 390S variant when subcultured (Byrd & Lyons, 1999). 390V, an isogenic rough revertant, then arose from 390S on subculture (Howard *et al.*, 2006). All three variants have stable phenotypes with low rates of spontaneous reversion (Byrd & Lyons, 1999; Howard *et al.*, 2006). We

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Abbreviations: GPL, glycopeptidolipid; MBC, minimum bactericidal concentration; NHS, normal human serum; NTM, non-tuberculous mycobacterium/mycobacteria; MDM, monocyte-derived macrophage; PIM, phosphatidyl-*myo*-inositol mannoside; TLR2, Toll-like receptor 2; TNF, tumour necrosis factor.

Three supplementary tables are available with the online version of this paper.

have demonstrated that the smooth colony phenotype of *M. abscessus* is due to expression of glycopeptidolipid (GPL) in the bacterial cell wall; GPL is minimally expressed by rough variants (Howard *et al.*, 2006). We have also demonstrated that the rough variants 390R and 390V are able to grow in human macrophages and stimulate macrophage Toll-like receptor 2 (TLR2), while the 390S variant lacks these capabilities (Byrd & Lyons, 1999; Howard *et al.*, 2006; Rhoades *et al.*, 2009). Unlike the rough variants, the 390S variant is able to form biofilms and exhibits sliding motility. We have postulated that this is due to GPL expression by 390S, which is supported by genetic studies demonstrating that GPL is necessary for both biofilm formation and sliding motility in *Mycobacterium smegmatis*, a non-pathogenic NTM (Recht *et al.*, 2000). We have provided evidence that expression of GPL facilitates the *M. abscessus* colonization phenotype, but 'masks' underlying bioactive cell-wall lipids involved in virulence (Rhoades *et al.*, 2009). In this study we have used genetic recombineering to delete the *M. abscessus* 390S *mmpL4b* gene, a gene coding for a membrane protein which has been found to play an essential role in GPL expression by NTM (Recht *et al.*, 2000; Ripoll *et al.*, 2007; Medjahed & Reyrat, 2009). Using this deletion mutant we provide direct evidence that GPL plays a role in both the colonization and invasion phenotypes of *M. abscessus*.

METHODS

Mycobacterial strains and preparation of bacterial stocks.

Strains used in the present study are listed in Supplementary Table S1, available with the online version of this paper. The *M. abscessus* 390S smooth colony variant was used as wild-type in these studies (Byrd & Lyons, 1999; Howard *et al.*, 2002, 2006; Rhoades *et al.*, 2009). Bacteria were maintained as titrated frozen stocks stored at -70°C with intermittent passage for 3 days on Middlebrook 7H11 agar plates supplemented with Middlebrook OADC (BD) followed by flash freezing. To prepare single-cell frozen bacterial stocks for experiments, lawns of the different bacterial strains were plated on Middlebrook 7H11 OADC agar plates and incubated at 37°C . After 3 days bacteria were harvested and placed into sterile Eppendorf tubes containing 1.0 ml sterile PBS and five glass beads. Tubes were pulse-vortexed 50 times, after which residual aggregates of bacteria were allowed to settle for 20 min. The top 500 μl of bacterial supernate was removed and the supernates from two to four tubes were pooled in 50 ml conical tubes. Bacteria were then sonicated on high power in a sonicating waterbath for 20 s to break apart any residual bacterial aggregates. The bacterial suspension was aliquoted into multiple Eppendorf tubes, which were then flash frozen and stored at -70°C . Individual tubes were thawed and titrated to determine c.f.u. for a particular frozen stock.

Construction of the *M. abscessus* *mmpL4b* mutant. *M. abscessus* 390S is a well-characterized smooth variant which expresses abundant GPL (Byrd & Lyons, 1999; Howard *et al.*, 2002, 2006; Rhoades *et al.*, 2009). The *mmpL4b* gene is a non-essential gene which is necessary for *M. abscessus* GPL production. In a prior study, this gene was used as a target to compare three different approaches for generating allelic-exchange mutants in *M. abscessus*. It was found that the recombineering system was the most efficient method for this purpose (Medjahed & Reyrat, 2009), and thus this was the approach used in our study. The plasmids and primers used are listed in

Supplementary Tables S2 and S3, respectively. The marked mutation construct of the *mmpL4b* gene (MAB_4115c) was generated by PCR. Primers *mmpL4b1*-F and *mmpL4b1*-Rv, containing *EcoRI* and *Sall* sites, respectively, were used to amplify a fragment (fragment 1) encoding N-terminal amino acids 1–3, along with a 908 bp sequence upstream of the *mmpL4b* gene. Similarly, primers *mmpL4b2*-F and *mmpL4b2*-Rv, containing *Sall* and *ApaI* sites, respectively, were used to amplify a fragment (fragment 2) encoding amino acids 985–987, along with a 950 bp region downstream of the *mmpL4b* gene. Fragments 1 and 2 were digested by either *EcoRI*/*Sall* or *Sall*/*ApaI* (Biolabs) and cloned in *EcoRI*-*ApaI*-digested pBluescript II SK(+) (Stratagene) to generate pBSK- Δ *mmpL4b*. The zeocin resistance cassette was amplified from pLYG204 Zeo (Howard *et al.*, 1995) using primers Zeo-F and Zeo-Rv, purified and digested by *Sall* and cloned into the *Sall* site of pBSK- Δ *mmpL4b* to generate pBSK- Δ *mmpL4b*::Zeo. This allelic-exchange substrate was used to perform mutagenesis in *M. abscessus* 390S.

Complementation of the *M. abscessus* *mmpL4b* mutant. The complemented strain was constructed as follows. The *mmpL4b* gene (MAB_4115c) with 300 bp upstream and downstream from the target gene was amplified by PCR using polymerase *Pfu* (Biolabs) and the primers *mmpL4b*-compF and *mmpL4b*-compRv, each containing a *XbaI* site (Supplementary Table S3). The genomic DNA of *M. abscessus* 390S was used as template. The PCR product was purified, digested with *XbaI* and cloned into plasmid pNBV1 (Howard *et al.*, 1995), which was previously digested by *XbaI*, dephosphorylated by alkaline phosphatase and purified to generate pNBV1-*mmpL4b*. This construct was electroporated into the mutant strain *M. abscessus* Δ *mmpL4b* and the transformants were selected on hygromycin ($1000\text{ }\mu\text{g ml}^{-1}$).

Sliding motility and biofilm formation. To assess sliding motility, a liquid culture of *M. abscessus* was dropped (10 μl) onto plates containing 7H9 medium (0.3% agar) without an added carbon source. Plates were incubated at 37°C and colony spread was assessed after 7 days.

To assess biofilm formation, bacteria were cultured in a Calgary biofilm device (MBEC Bioproducts) (Ceri *et al.*, 1999) at a concentration of 2×10^4 c.f.u. in 200 μl Iscove's medium (Gibco) per well. The culture was incubated at 37°C on a shaker set to 100 r.p.m. and the medium was replaced every 24 h for a total culture time of 72 h. The biofilm pegs attached to the device lid were then placed in a new 96-well plate with 200 μl Iscove's medium in each well and left for 2 min as a wash step. Pegs were then removed from the lid (four pegs were harvested per bacterial strain), placed in tubes containing 1 ml sterile PBS, and sonicated for 20 s. Bacterial c.f.u. were then titrated on 7H11 agar plates.

***M. abscessus* infection of human macrophages.** Human mononuclear cells were isolated from buffy coats purchased from United Blood Services and cultured for 48 h in Teflon jars in Iscove's/5% NHS medium at 37°C in 5% CO_2 to facilitate monocyte maturation. Human monocytes were isolated by adherence to tissue culture wells (Falcon) followed by incubation for 24 h at 37°C in medium. Immediately prior to challenge with bacteria, monocyte-derived macrophage (MDM) monolayers were washed twice to remove non-adherent cells. MDM monolayers were then infected with 5.0×10^5 *M. abscessus* c.f.u. ml^{-1} in 0.5 ml Iscove's/5% NHS. At the time the infecting inocula of the different variants were added to wells, infecting inocula from the same stock tubes were also placed on 7H11 plates containing zeocin at a concentration of $50\text{ }\mu\text{g ml}^{-1}$. After 90 min incubation, the MDM monolayers were washed three times with 0.5 ml Iscove's/5% NHS to remove remaining extracellular bacteria, and 0.5 ml Iscove's/5% NHS added back to each well. Cell lysates containing bacteria were harvested at time 0 and

c.f.u. plated as described previously (Howard *et al.*, 2006). At 24 h, MDM monolayers were washed twice with 0.5 ml Iscove's/5 % NHS to remove nonadherent cells and extracellular bacteria. Cell lysates were plated and 0.5 ml Iscove's/5 % NHS was added back to remaining wells. This was repeated at 48 and 72 h, with a final plating of cell lysates at 72 h. We have found that NHS is a necessary component of wash medium as it prevents non-specific adherence of *M. abscessus* GPL-expressing variants to tissue culture wells (Greendyke & Byrd, 2008). Non-specific adherence of bacteria to tissue culture wells can lead to overgrowth of the bacteria in tissue culture media and spurious results. Progression of infection in MDM monolayers is dependent on *M. abscessus* spreading from initially infected macrophages to uninfected macrophages as occurs with intracellular bacterial pathogens such as *Legionella pneumophila* (Byrd & Horwitz, 1989). Although *M. abscessus* can multiply to a limited extent in tissue culture medium, extensive washing of monolayers after initial infection and at time points throughout the experiment minimizes extracellular growth as an artefact (Greendyke & Byrd, 2008). In prior studies we have demonstrated that after extensive washing of MDMs to reduce extracellular bacteria, the addition of amikacin to kill any residual extracellular bacteria does not change the outcome of macrophage infection comparing the 390R, 390V and 390S *M. abscessus* variants (Howard *et al.*, 2006; Greendyke & Byrd, 2008). To assess whether addition of amikacin has an effect on the growth of the *mmpL4b* deletion mutant in MDMs we also compared washing alone to washing followed by the addition of amikacin for 6 h at a concentration of 64 µg ml⁻¹, the minimum bactericidal concentration (MBC) for the 390 variants (Greendyke & Byrd, 2008), prior to plating cell lysates for time 0 c.f.u. Middlebrook 7H9 broth (Difco) was used for dilution of cell lysates prior to plating for c.f.u. Middlebrook 7H11 agar (Difco) plates, with and without zeocin, were used for plating c.f.u. from infected monolayers. In addition, nuclear counts in replicate wells were determined, and c.f.u. standardized to 10⁵ nuclei to account for any differences in monocyte number in the different monolayers, as has been described previously (Byrd, 1997). Replicate monolayers treated in the same fashion were fixed with PBS/4 % paraformaldehyde and photographed with a Nikon TE1 2000 U phase-contrast microscope.

Induction of macrophage TNFα release by *M. abscessus* variants. Human monocytes were isolated, cultured in Teflon jars to generate MDMs, and plated in tissue culture wells as described above. After 24 h incubation in medium at 37 °C in 5 % CO₂, the tissue culture wells containing MDM monolayers were washed three times with 0.5 ml Iscove's medium, and 0.5 ml Iscove's medium without NHS was added back to each well. Mouse anti-human TLR2 (eBioscience clone T2.5) (10 µg ml⁻¹) and mouse IgG₁ isotype control (eBioscience) (10 µg ml⁻¹) were added to appropriate wells and incubated for 1 h at 37 °C in 5 % CO₂. Wells were then infected with 2.5 × 10⁶ *M. abscessus* c.f.u. per well (bacteria to macrophage ratio in the range of 10 to 1), and incubated at 37 °C, 5 % CO₂ for 2 h. Supernates were filtered through 0.22 µm filter units and stored at -70 °C for later determination of TNFα levels. TNFα levels were measured using the TNFα ELISA BD OptEIA (BD Biosciences). In addition, nuclear counts in replicate wells were determined at each time point, and TNFα was standardized to 10⁵ nuclei to account for any differences in macrophage number in monolayers subjected to different experimental treatments or conditions (Rhoades *et al.*, 2009). We have found that this is critical for meaningful interpretation of results since it allows for standardization among different experimental groups. In addition, there is substantial variation in tissue culture adherence of MDMs from different donors; thus this also allows for meaningful comparisons between experiments.

GPL extraction and analysis. *M. abscessus* lipids were extracted from plate-grown cultures as previously described (Howard *et al.*,

2006). Briefly, cultures were extracted with CHCl₃/CH₃OH [2:1, v/v; 10 ml (g wet weight bacteria)⁻¹]. Bacteria were extracted twice at 56 °C (15 min with sonication) and once at 4 °C overnight, and insoluble material was removed by centrifugation and filtration (0.2 µm PTFE filter; Millipore). Combined extracts were subjected to biphasic partitioning in CHCl₃/CH₃OH/H₂O (4:2:1, by vol.). Lipids in the organic phase were stored at -20 °C. Total lipid extracts were resuspended at 10 µg ml⁻¹ in CHCl₃/CH₃OH and spotted onto aluminium-backed silica gel-60 TLC plates (EM Science). Lipids were resolved in CHCl₃/CH₃OH/H₂O (100:14:0.8, by vol.). Plates were sprayed with 1-naphthol (Sigma; 3 % with 10 % H₂SO₄ in CH₃OH) to identify characteristic GPL spots associated with *M. abscessus* 390S (Howard *et al.*, 2006).

Statistics. Data were compared using Student's *t* test. Differences were considered significant with *P* < 0.05.

RESULTS

Generation of a *M. abscessus* 390S *mmpL4b* deletion mutant

To further define the role of GPL in *M. abscessus* pathogenesis, we used genetic recombineering to generate a deletion mutant deficient in GPL synthesis from our 390S variant. We targeted the *mmpL4b* gene, a non-essential gene that plays a key role in GPL synthesis and which has previously been demonstrated to be a suitable target for generating a *M. abscessus* GPL-deficient mutant (Medjahed & Reyrat, 2009). After bacterial transformation and plating, a *M. abscessus* 390S derivative colony with the rough phenotype was picked for further analysis. To confirm loss of *mmpL4b*, PCR was performed using primer sets flanking *mmpL4b3-F* and *mmpL4b3-R* (Fig. 1) (Supplementary Table S3). Wild-type 390S yielded a 2960 bp product, while the rough variant yielded a 560 bp product consistent with loss of the *mmpL4b* gene. In addition, a Southern blot hybridized with a DNA probe overlapping the *mmpL4b* gene generated different banding patterns comparing wild-type 390S and the rough variant (Fig. 2). Consistent with the rough phenotype (Fig. 3), these results confirm loss of *mmpL4b* in the transformant. We have previously characterized GPL expression of wild-type 390S by TLC (Howard *et al.*, 2006). TLC analysis of wild-type 390S demonstrated characteristic GPL spots while 390S Δ*mmpL4b* demonstrated minimal GPL expression, consistent with loss of *mmpL4b* (Fig. 3). A complemented strain was generated by incorporating the construct pNBV1-*mmpL4b*, carrying 300 bp upstream and downstream of the *mmpL4b* gene, into the *M. abscessus* 390S Δ*mmpL4b* mutant. This variant exhibited the smooth phenotype and expressed GPL (Fig. 3).

390S Δ*mmpL4b* has lost sliding motility

Both sliding motility and biofilm formation are believed to facilitate survival of NTM as environmental saprophytes. GPL in *M. smegmatis* has been demonstrated to be essential for sliding motility and biofilm formation (Recht *et al.*,

Fig. 1. (a) PCR analysis of the *mmpL4b* locus in *M. abscessus* 390S wild-type (lane S) and the *mmpL4b* knockout strain (lane R). Lane M, size markers. (b) Schematic representation of the wild-type *mmpL4b* locus and the allelic-exchange mutant with the zeocin resistance cassette (Zeo RC). The predicted phenotype is indicated in parentheses: S, smooth; R, rough.

2000). In addition, the presence of GPL in *M. abscessus* has been correlated with the ability of different variants to form biofilm and exhibit sliding motility (Howard *et al.*, 2006). Unlike wild-type 390S and the complemented strain, 390S $\Delta mmpL4b$ did not exhibit sliding motility or biofilm formation (Fig. 4a, b). *M. abscessus* 390V is a naturally occurring spontaneous mutant derived from 390S which has lost the ability to express GPL, and is thus an ideal comparator strain for 390S $\Delta mmpL4b$. When compared in the same experiments for biofilm formation and sliding motility, 390V and 390S $\Delta mmpL4b$ did not form biofilms or exhibit sliding whereas wild-type 390S did so (Fig. 4c and not shown). These results conclusively demonstrate a critical role for GPL in *M. abscessus* sliding motility and biofilm formation.

390S $\Delta mmpL4b$ has acquired the ability to stimulate human macrophage TLR2

We have previously demonstrated that our rough *M. abscessus* variants lacking GPL (390R and 390V) stimulate the human macrophage innate immune response through TLR2 stimulation. At least one class of the *M. abscessus* cell-wall ligands mediating this interaction are the phosphatidyl-*myo*-inositol mannosides (PIMs). Although the intact 390S variant does not stimulate TLR2 signalling in human macrophages, 390S from which GPL has been selectively removed acquires the ability to stimulate TLR2. Since 390S, 390R and 390V all possess similar PIMs in their

cell walls capable of stimulating TLR2, this led to the conclusion that GPL is masking underlying PIMs in the *M. abscessus* 390S cell wall, preventing their interaction with human macrophage TLR2 (Rhoades *et al.*, 2009).

We tested the ability of the 390S $\Delta mmpL4b$ deletion mutant to stimulate human macrophage TLR2. We have previously demonstrated the specificity of the *M. abscessus*-TLR2 interaction in experiments using HEK cells transfected with TLR2, and in TLR2-blocking antibody experiments (Rhoades *et al.*, 2009). Here we demonstrate that 390S $\Delta mmpL4b$ acquires the ability to stimulate human macrophage TLR2 to a similar extent as our comparator strain 390V (Fig. 5). These results, taken with our previous study (Rhoades *et al.*, 2009), demonstrate that GPL is masking underlying *M. abscessus* 390S ligands that bind to the human macrophage TLR2 receptor.

390S $\Delta mmpL4b$ has acquired the ability to replicate in human macrophages

The rough *M. abscessus* variants we have characterized which lack GPL (390R and 390V) are able to replicate in human macrophages, but *M. abscessus* 390S is not. Our data indicate that GPL masks underlying bioactive cell-wall lipids; exposure of these lipids on the surface of *M. abscessus* may be necessary for intracellular replication in macrophages to occur. To test this hypothesis we examined the ability of 390S $\Delta mmpL4b$ to replicate in human monocyte-derived macrophages (MDMs). The deletion

Fig. 2. (a) Southern blot analysis of the *mmpL4b* locus in *M. abscessus* 390S wild-type (lane 1) and the allelic-exchange mutant (lane 2) using the upstream part of *mmpL4b* as a probe. (b) Schematic representation of the wild-type *mmpSL4* operon in *M. abscessus* 390S (1) and in the allelic-exchange mutant (2). The predicted phenotype is indicated in parentheses: S, smooth; R, rough.

Fig. 3. Characterization of 390S $\Delta mmpL4b$ and the complemented strain. Photographs of 3-day-old *M. abscessus* colonies grown at 37 °C at the top of figure demonstrate the smooth colony phenotype of wild-type strain 390S (WT), the rough colony morphology of *mmpL4b* deletion mutant ($\Delta mmpL4b$) and restoration of the smooth phenotype in complemented mutant ($\Delta mmpL4b$ comp). Corresponding TLC of extracted lipids (with detection by 1-naphthol) demonstrates characteristic *M. abscessus* GPL species which are absent in the *mmpL4b* deletion mutant.

mutant replicated over 100-fold in MDMs while wild-type 390S exhibited minimal growth (Fig. 6a). Infection of MDM monolayers with 390S $\Delta mmpL4b$ was accompanied by extensive macrophage aggregation beginning at 24 h, accompanied by progressive destruction of monolayers (Fig. 6b). Formation of infected cellular aggregates has been previously reported during MDM monolayer infection with *M. abscessus* rough variants, as well as with virulent *M. tuberculosis* (Byrd, 1997; Byrd & Lyons, 1999). The complemented strain did not differ in terms of replication over 24 h compared to wild-type; however, by 48 h intracellular replication had begun to occur (Fig. 6a). This was likely due to loss of the plasmid containing *mmpL4b* during the course of the experiment. This is supported by our observation that the percentage of *M. abscessus* rough variant c.f.u. recovered from macrophage lysates infected with the complemented mutant increased dramatically at 48 h (9 %) and 72 h (37 %), compared to 0 and 24 h (0 %). This was accompanied by formation of MDM aggregates beginning at 48 h (Fig. 6b), although not nearly as extensive as that seen in MDM monolayers infected with 390S $\Delta mmpL4b$. To ensure that there was no cross-contamination of our tissue culture experiments with *M. abscessus* wild-type rough variants, which we also use in

Fig. 4. Loss of colonization capability by *M. abscessus* 390S $\Delta mmpL4b$. (a) *M. abscessus* 390S $\Delta mmpL4b$ has lost sliding motility capability. 390S wild-type, 390S *mmpL4b* deletion mutant ($\Delta mmpL4b$) and the complemented mutant ($\Delta mmpL4b$ comp) were plated on motility agar and sliding motility was compared at 7 days. (b) *M. abscessus* 390S $\Delta mmpL4b$ has lost biofilm-forming capability. Strains 390S wild-type, $\Delta mmpL4b$ and $\Delta mmpL4b$ comp were assessed for their ability to form biofilm in a Calgary biofilm device, as described in Methods; c.f.u. in supernates recovered from the peg inserts were determined at 72 h. Data are means $\pm$ SEM of two experiments, each done in triplicate. *390S $\Delta mmpL4b$ versus 390S wild-type and 390S $\Delta mmpL4b$ comp; $P < 0.01$, *t*-test. (c) *M. abscessus* 390S $\Delta mmpL4b$ biofilm capability is comparable to that of the 390V variant. The biofilm-forming capability of 390S $\Delta mmpL4b$ was compared to that of 390S wild-type and the naturally occurring 390V variant, using the assay as in (b). Data are means $\pm$ SD of duplicate determinations. *390S $\Delta mmpL4b$ and 390V versus 390S wild-type; $P < 0.05$, *t*-test.

our laboratory, we plated c.f.u. at all time points on 7H11 agar with and without zeocin. Whereas c.f.u. from the wells infected with 390S $\Delta mmpL4b$ or complemented 390S $\Delta mmpL4b$ had similar numbers of c.f.u. on both plates at all time points, the 390S wild-type grew only on the 7H11 plates without zeocin, confirming the origin of the *M. abscessus* variants throughout the course of the infection experiment. In addition, 390S $\Delta mmpL4b$ multiplied to a similar extent in MDM monolayers as the comparator strain 390V (Fig. 6c). Our assay is performed by washing MDM monolayers extensively after initial infection to remove extracellular bacteria so as to assess intracellular growth. We have previously found no difference in growth

Fig. 5. (a) *M. abscessus* 390S $\Delta mmpL4b$ has acquired the ability to stimulate human MDMs comparable to that induced by the 390V variant. Human MDMs were challenged with 390S wild-type (WT), 390S *mmpL4b* deletion mutant ($\Delta mmpL4b$), the complemented mutant ($\Delta mmpL4b$ comp) and the comparator strain 390V. Culture supernates were collected after 2 h and assayed by ELISA for TNF α . Data are means $\pm$ SEM of two experiments done in duplicate. *390S $\Delta mmpL4b$ and 390V versus 390S wild-type and 390S $\Delta mmpL4b$ comp; $P < 0.01$, *t*-test. (b) TNF α release from human macrophages in response to 390S $\Delta mmpL4b$ is blocked by antibody to TLR2. Human MDMs were preincubated with antibody to TLR2 or isotype control antibody and then challenged with *M. abscessus* 390S $\Delta mmpL4b$. Culture supernates were collected 2 h after addition of bacteria and assayed by ELISA for TNF α . Data are means $\pm$ SEM of two experiments done in duplicate. *TLR2 versus no-antibody and isotype control antibody; $P < 0.01$, *t*-test.

of rough and smooth strains in MDM monolayers when comparing extensive washing to extensive washing plus addition of amikacin at the MBC for 6 h after initial

infection (Howard *et al.*, 2006; Greendyke & Byrd, 2008). Results comparing wild-type 390S to 390S $\Delta mmpL4b$ and 390V were identical when amikacin at the MBC was added immediately after infection for 6 h to ensure killing of extracellular bacteria (Fig. 6d). Thus, these results indicate that a single genetic change associated with loss of an outermost bacterial cell-wall lipid (GPL) is sufficient to convert *M. abscessus* to a phenotype able to replicate within human macrophages.

DISCUSSION

In this study we have demonstrated loss of GPL expression by the *M. abscessus* 390S variant through deletion of the *mmpL4b* gene, which codes for a membrane protein that is critical for GPL expression. To our knowledge, our study is the first to demonstrate that loss of a molecule at the outermost surface of the cell wall converts a bacterium to an intracellular replicative phenotype. In contrast to our findings, other studies have demonstrated that mycobacterial gene deletion mutants generally have reduced pathogenicity and/or ability to replicate in macrophages. For example, deletion of the *ompATb* gene of *M. tuberculosis*, a gene coding for a putative porin, leads to reduced intracellular replication in macrophages, possibly as a result of diminished ability to respond to reduced phagosomal pH (Raynaud *et al.*, 2002). In addition, using a genetic approach, we confirm the results of our prior study, which was the first to provide evidence that a cell-wall lipid (GPL) can mask underlying immunostimulatory cell-wall lipids (Rhoades *et al.*, 2009).

The explanation for our observations relates to the biological inertness of *M. abscessus* GPL and the concomitant masking of bioactive cell-wall molecules by GPL in the *M. abscessus* cell wall. It has been demonstrated that GPLs are localized to the outermost portion of the mycobacterial cell envelope of NTM (Barrow *et al.*, 1980), and are responsible for the smooth phenotype which occurs in species of NTM where there are smooth/rough colony variants (Eckstein *et al.*, 2000; Howard *et al.*, 2006). The GPL molecule consists of a tripeptide-amino alcohol core with an amide-linked long-chain fatty acid. This lipopeptide core is substituted with 6-deoxytalose and O-methylated rhamnose to generate the non-specific core GPLs (nsGPLs) (Brennan & Goren, 1979; Ripoll *et al.*, 2007). This is the form of GPL present in the *M. abscessus* cell wall. We have previously demonstrated that nsGPL of *M. abscessus* is not strongly immunogenic (Rhoades *et al.*, 2009). While the nsGPLs of *M. abscessus*, *M. chelonae* and *M. smegmatis* are structurally identical (Ripoll *et al.*, 2007), the variable haptenic oligosaccharide at 6-deoxytalose present in *M. avium-intracellulare* GPL forms the basis for serotyping *M. avium-intracellulare* strains and is felt to bestow immunogenic properties on serovar-specific GPLs (ssGPLs) (Brennan & Nikaido, 1995). It has been reported that ssGPLs from smooth variants of *M. avium-intracellulare* serovars 1 and 2 stimulate macrophage TNF α release

Fig. 6. *M. abscessus* 390S $\Delta mmpL4b$ has acquired the ability to replicate in human macrophages. (a) Growth of *M. abscessus* 390S $\Delta mmpL4b$ (▼) compared to 390S wild-type (●) and complemented 390S $\Delta mmpL4b$ (▲). Bacterial c.f.u. in MDM monolayers were determined at the indicated intervals and corrected to c.f.u. per 10^5 MDM. Monolayers were >98 % viable at time 0 immediately after infection as assessed by trypan blue exclusion. Data are means $\pm$ SEM of two experiments done in duplicate. ** $\Delta mmpL4b$ versus wild-type and complemented $\Delta mmpL4b$; $P < 0.01$, t -test. *Complemented $\Delta mmpL4b$ versus wild-type; $P < 0.01$, t -test. (b) Progression of macrophage infection. Replicate MDM monolayers were fixed and photographed at 0 (immediately after infection), 24 and 48 h to monitor the effect of infection with the *M. abscessus* variants. Original image taken at $\times 100$ magnification. (c) Growth of *M. abscessus* 390S $\Delta mmpL4b$ (▼) compared to 390S wild-type (●) and the 390V comparator strain (◆). Bacterial c.f.u. in MDM monolayers were determined at the indicated intervals and corrected to c.f.u. per 10^5 MDM. Monolayers were >98 % viable at time 0 immediately after infection as assessed by trypan blue exclusion. Data are means $\pm$ SD of duplicate determinations. *390S $\Delta mmpL4b$ and 390V versus 390S wild-type; $P < 0.05$, t -test. (d) Growth of *M. abscessus* 390S $\Delta mmpL4b$ (▼) compared to 390S wild-type (●) and the 390V comparator strain (◆) with addition of amikacin immediately after infection. The experiment was performed as in (c) except that amikacin at a concentration of $64 \mu\text{g ml}^{-1}$ (MBC) was added for 6 h immediately after infection prior to plating c.f.u. at time 0. Monolayers were >98 % viable at time 0 immediately after infection as assessed by trypan blue exclusion. Data are means $\pm$ SD of duplicate determinations. *390S $\Delta mmpL4b$ and 390V versus 390S wild-type; $P < 0.05$, t -test.

via TLR2, while nsGPLs do not (Sweet & Schorey, 2006). Thus, in contrast to *M. avium-intracellulare* ssGPL, *M. abscessus* nsGPL is a relatively inert molecule in terms of its ability to stimulate the host innate immune response. However, in addition to nsGPLs, *M. abscessus* possesses mannose-containing bioactive cell-wall lipids (Rhoades *et al.*, 2009). We have previously demonstrated the presence of one of these classes of molecules known as the phosphatidyl-*myo*-inositol mannosides (PIMs) in the *M. abscessus* cell wall (Rhoades *et al.*, 2009). PIMs isolated from *M. tuberculosis* are macrophage TLR2 agonists

(Krutzik & Modlin, 2004) and are also involved in intracellular trafficking of this pathogen (Chua *et al.*, 2004). We have also demonstrated that identical *M. abscessus* PIMs are present in the 390R and 390V rough variants, and in the 390S smooth variant used in this study. Although 390R and 390V stimulate human macrophage TLR2, 390S does not. However, when GPL is selectively removed from the 390S surface this variant gains the ability to stimulate TLR2. Furthermore, PIMs isolated from all three variants and coated onto polystyrene beads are able to stimulate human macrophage TLR2. Finally, mannose-

binding lectins bind avidly to the surface of the 390R and 390V variants, but not 390S, suggesting that the PIMs found in this variant are masked by GPL in the outermost cell wall (Rhoades *et al.*, 2009). In addition to PIMs, other biosynthetically related mannosylated lipoglycoconjugates such as lipomannan (LM) and mannose-capped lipoarabinomannan (LAM) play important roles in *M. tuberculosis* pathogenesis (Schlesinger *et al.*, 1994; Torrelles *et al.*, 2006). *M. abscessus* possesses several orthologues (MAB_1991c, MAB_2689, MAB_0205c) of genes identified as being directly or indirectly involved in synthesis of these glycolipids (Kaur *et al.*, 2007; Scherman *et al.*, 2009; Driessen *et al.*, 2010); it is thus likely that these molecules are also present in *M. abscessus*.

Since our previous studies utilized naturally occurring *M. abscessus* variants, it could be argued that the spontaneous changes in GPL expression associated with rough/smooth phenotypic variation are accompanied by other changes which affect biofilm forming capability, sliding motility, immunostimulatory activity and the ability to replicate in macrophages. In this study we conclusively demonstrate that deletion of the gene *mmpL4b*, which is essential for GPL synthesis and transport to the cell surface, is sufficient for *M. abscessus* 390S to acquire an invasive, immunostimulatory phenotype.

Our results support emerging clinical data which indicate that the presence or absence of GPL is associated with human pathogenic potential of *M. abscessus*. The rough colony phenotype has been associated with fatal disseminated infection (Sanguinetti *et al.*, 2001). Epidemiological data indicate that the rough *M. abscessus* colony phenotype is the predominant form isolated from patients with clinical lung disease and/or chronic colonization, whereas the smooth form is isolated from those who are transiently colonized without symptoms and/or from patients with wound infection secondary to contact with contaminated environmental sources (Jönsson *et al.*, 2007). In addition, a recent clinical report documented that deterioration of lung function in a *M. abscessus*-infected patient was associated with conversion of sputum isolates from the smooth phenotype to an isogenic rough phenotype (Catherinot *et al.*, 2009).

Analysis of the recently published *M. abscessus* genome has demonstrated a number of genes which are shared in common with two of the pathogens most frequently isolated from the lungs of cystic fibrosis patients – *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Ripoll *et al.*, 2009). It is postulated that these genes were acquired from distantly related environmental bacteria via horizontal gene transfer, and there is evidence that the proteins encoded by these genes facilitate colonization of the respiratory tract of cystic fibrosis patients (Ripoll *et al.*, 2009). These shared genes may thus facilitate colonization of the respiratory tract by *M. abscessus* GPL-expressing smooth variants in patients with cystic fibrosis by promoting extracellular survival in biofilms. As we have

noted, rough, non-GPL-expressing *M. abscessus* variants are capable of invading and replicating in macrophages (Byrd & Lyons, 1999; Howard *et al.*, 2006; Greendyke & Byrd, 2008) and respiratory epithelial cells (unpublished observations). Other identified genes in the *M. abscessus* genome have homology to genes known to play a role in the virulence of the intracellular pathogen *M. tuberculosis* (Ripoll *et al.*, 2009). It is likely that expression of these genes by *M. abscessus* rough variants promotes intracellular survival in macrophages and facilitates invasion into the lung parenchyma. Thus, with recently identified genes facilitating both extra- and intracellular survival, *M. abscessus* is uniquely adapted to survive both in the lung airways and within host cells in the lung parenchyma. The results of our study indicate that the presence or absence of GPL may be the critical determinant in the outcome of *M. abscessus* lung infection.

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