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Disruption of Mycobacterial AftB Results in Complete Loss of Terminal $\beta(1 \rightarrow 2)$ Arabinofuranose Residues of Lipoarabinomannan

Monika Jankute,† Luke J. Alderwick,‡ Stephan Noack,‡ Natacha Veerapen,† Jérôme Nigou,$^§$ and Gurfyal S. Besra*†

†School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, B15 2TT Birmingham, United Kingdom
‡Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, Jülich D-52425, Germany
§Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, 31077 Toulouse, France

Supporting Information

ABSTRACT: Lipoarabinomannan (LAM) and arabinogalactan (AG) are the two major mycobacterial cell wall (lipo)-polysaccharides, which contain a structurally similar arabinan domain that is highly branched and assembled in a stepwise fashion by variety of arabinofuranosyltransferases (Ara/T). In addition to playing an essential role in mycobacterial physiology, LAM and its biochemical precursor lipomannan possess potent immunomodulatory activities that affect the host immune response. In the search of additional mycobacterial Ara/Ts that participate in the synthesis of the arabinan segment of LAM, we disrupted aftB (MSMEG_6400) in Mycobacterium smegmatis. The deletion of chromosomal aftB locus could only be achieved in the presence of a rescue plasmid carrying a functional copy of aftB, strongly suggesting that it is essential for the viability of M. smegmatis. Isolation and detailed structural characterization of a LAM molecule derived from the conditional mutant deficient in AftB revealed the absence of terminal $\beta(1 \rightarrow 2)$-linked arabinofuranosyl residues. Furthermore, we demonstrated that truncated LAM displays proinflammatory activity, which is due to its ability to activate Toll-like receptor 2. All together, our results indicate that AftB is an essential mycobacterial Ara/T that plays a role in the synthesis of the arabinan domain of LAM.

Despite the existence of treatments for tuberculosis (TB), TB continues to represent a major healthcare challenge, accounting for nearly 9 million new infections and over 1 million deaths each year. Indeed, this global health threat is escalating, given the variable efficacy of the vaccine strain BCG, the increased susceptibility of HIV-infected individuals to TB, and the increased prevalence of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis, the causative agent of the disease. As current treatments lose their efficacy, it is vital that we address the increasing health burden of TB by replenishing the drug pipeline with new drugs, drug targets, and improved treatment regimes.

The cell envelope of M. tuberculosis is unique in that it contains a thick carbohydrate and lipid rich layer that accounts for its inherent resistance to numerous drugs and contributes to its persistence and virulence. This is due to the key cell envelope structures, notably the mycolyl-arabinogalactan-peptidoglycan complex (mAGP), lipomannan (LM), and lipoarabinomannan (LAM). The latter glycolipids, in addition to their structural roles, exhibit potent immunomodulatory activities and thus are probably important modulators of the host—pathogen interactions during the course of infection.

In mycobacteria, LM is composed of approximately 20–25 $\alpha(1 \rightarrow 6)\alpha-D$-mannopyranosyl (Manp) residues connected in a linear fashion and are anchored to the inner and outer membrane via their phosphatidyl-myo-inositol (PI) unit. This mannose core is further decorated with 7–10 singular $\alpha(1 \rightarrow 2)$ Manp residues. The mature LM then serves as a precursor to the formation of LAM where it is further glycosylated by large arabian domain consisting of approximately 55–70 Araf residues in a linear $\alpha(1 \rightarrow 5)-\alpha-D$-Araf fashion with 3,5-$\alpha-D$-Araf branches (Figure 1). Recent studies suggest that this single arabian chain is attached to the mannan core at the O-6 position of the mannosyl residue and not O-2 as previously reported. Although, the arabian structure of LAM is more variable than that of AG, two types of highly branched and conserved motifs remain. These are the tetra-arabinoside ($\beta-D$-Araf(1 $\rightarrow$ 2)-$\alpha-D$-Araf(1 $\rightarrow$ 5)-$\alpha-D$-Araf(1 $\rightarrow$ 5)-$\alpha-D$-Araf) and hexa-arabinoside (($\beta-D$-Araf(1 $\rightarrow$ 2)-$\alpha-D$-Araf)$_2$-$3,5$-$\alpha-D$-Araf(1 $\rightarrow$ 5)-$\alpha-D$-Araf) motifs, both of which end with a characteristic disaccharide unit (Araf-$\beta(1 \rightarrow 2)$-Araf-$\alpha(1 \rightarrow)$). The termini

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of the arabinan domain are completed with the capping motifs that are species dependent. The fast growers, such as *M. smegmatis*, are capped by phosphoinositide units (PILAM). The slow growers including *M. tuberculosis* are capped with 2–3 mannose residues (Man-LAM), and some species, such as *Mycobacterium chelonae* have uncapped LAM (AraLAM). In addition, *C. glutamicum*, a closely related organism to *M. tuberculosis* and a desired model organism to study mycobacterial AG and LM assembly, produces LAM that lacks a highly branched arabinan domain and contains single Ara residues attached to the mannan core by AftE. As a result, although the structure of LAM has been fairly well described, understanding physiological functions and biological activities of lipoglycans prove to be challenging.

Figure 1. Structural representation of the wild type lipoarabinomannan and the unknown arabinofuranosyltransferase investigated in this study.

Construction and Growth of the *M. smegmatis* *aftB* Conditional Mutant. Our earlier studies demonstrated that the nonessential AftB from *C. glutamicum* (Cg-AftB) adds the terminal β(1 → 2)-linked Ara residues to the arabinan domain of AG before its decoration with mycolic acids. In this study, we investigated the potential role of AftB in the biosynthesis of LAM in *M. smegmatis*. For this purpose, we constructed a knockout phage pMSMEG6400 designed to replace the chromosomal *M. smegmatis*Δ*aftB::pMV306-aftB* mutant. We have shown, for the first time, that AftB has a dual function and is responsible for β(1 → 2)-linked Araf incorporation into LAM. Moreover, we have analyzed the immunological properties of a LAM molecule isolated from the *aftB* conditional mutant in order to shed light on the structure–function relationship between mycobacterial lipoglycans and host pattern recognition receptors (PRRs), notably Toll-like receptor 2 (TLR2).

## RESULTS AND DISCUSSION

### Results and Discussion

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Previous studies employing high density mutagenesis studies suggest that Rv3805c in *M. tuberculosis* is an essential gene.\(^{17}\) It is reasonable to conclude that *AftB* is essential for *M. tuberculosis* viability and therefore represents an attractive drug target.

**Initial Characterization of Lipoglycans Extracted from the *M. smegmatis* *AftB* Conditional Mutant.**

Strains of *M. smegmatis*, *M. smegmatis*::pMV306-*aftB*, and conditional *M. smegmatis*Δ*aftB*::pMV306-*aftB* mutant grown in the presence and absence of acetamide were examined for their ability to synthesize LAM. Exponentially growing cultures including *M. smegmatis*Δ*aftB*::pMV306-*aftB* depleted of *AftB* were labeled with \([1, 2-^{14}C]\)-glucose for 10 h followed by lipoglycan extraction. \([^{14}C]\)-LAM and \([^{14}C]\)-LM were profiled using SDS-PAGE analysis and the lipoglycans revealed and quantified by phosphorimaging (Figure 3a). Extracts from all strains showed the presence of \([^{14}C]\)-LAM and \([^{14}C]\)-LM (Figure 3a). Densitometry analysis revealed no significant difference in lipoglycan profiles extracted from different strains. Subsequently, \([^{14}C]\)-lipoglycans were assessed for the reactivity with an anti-AraLAM (α-AraLAM) monoclonal antibody (mAb, F30-5), which recognizes the terminal arabinan branches of LAM.\(^{18, 19}\) All lipoglycan preparations reacted with the antibody F30-5, with an exception of LAM isolated from the *M. smegmatis*Δ*aftB*::pMV306-*aftB* depleted of *AftB* (Figure 3b). These results strongly indicate that AftB is involved in the synthesis of the nonreducing end of arabinan domain of LAM in *M. smegmatis*.

**Structural Characterization of LAM Isolated from the *M. smegmatis* *AftB* Conditional Mutant.** One of the disadvantages in generating conditional mutants by employing...
CESTET is the restricted time scale where cells have to be cultured in the absence of acetamide long enough to deplete the intracellular protein of interest but short enough not to yield persister cells, which results in a modest yield of cell mass. In order to further analyze the different LAM preparations by NMR spectroscopy and improve the carbon signal intensity due to poor cell yield, we labeled M. smegmatis ΔaftB pMV306-afIB cultured in the presence and absence of acetamide with [13C]-glucose for 10 h, followed by lipoglycan extraction. Purified [13C]-lipoglycans were recovered from the crude extracts by hydrophobic and gel exclusion chromatography before subjecting samples to 1H-13C HSQC analysis. On the basis of our earlier studies with mycobacterial LAM and previously published work, we assigned the proton and carbon resonances of the different spin systems using 1H-13C HSQC experiments. The 13C resonances at δ101.1 ppm and δ102.3 ppm correlating to protons at δ5.11 ppm and δ4.91 ppm were assigned as 2,6-α-Manp and 6-α-Manp linkages, respectively (Figure 4a,b). The resonance at δ105.0 ppm, which correlated to an anomic proton at δ5.04 ppm, was designated as t-α-Manp. As expected, the resonances associated with the mannann core (2,6-α-Manp, 6-α-Manp, and t-α-Manp) remained unaffected by the disruption of AftB activity and as a result were visible in both 1H-13C HSQC spectra (Figure 4a,b). The resonances associated with the arabinan domain, however, were notably more complex in the spectra of LAM isolated from the M. smegmatis ΔaftB::pMV306-afIB grown in the presence of acetamide (Figure 4a) than compared to the resonances of LAM extracted from M. smegmatis ΔaftB::pMV306-afIB grown in the absence of the inducer (Figure 4b). Several spin systems with 13C resonances at δ110.0 ppm and δ110.4 ppm were assigned to anomic protons at δ5.16 ppm and δ5.08 ppm, respectively, and designated as 5-α-Araf in different chemical environments. The 3,5-α-Araf residues corresponded to δ110.4 ppm and were assigned to protons at δ5.09 ppm. The 2,3-α-Araf and 2,5-α-Araf linkages corresponded to δ108.4 ppm and δ108.6 ppm with protons at δ5.24 ppm and δ5.17 ppm, respectively (Figure 4a). Finally, the 13C resonance at δ103.5 ppm was designated to δ 5.14 ppm as the t-β-Araf linkage (Figure 4a). Most importantly, only 5-α-Araf and 3,5-α-Araf residues were conserved in the LAM of M. smegmatis ΔaftB::pMV306-afIB depleted of AftB, but 2,3-α-Araf, 2,5-α-Araf, and t-β-Araf linkages were absent due to the loss of β(1 → 2) linkages (Figure 4a,b). The 1H-13C HSQC experiments confirmed that AftB acts as a β(1 → 2) Ar/T in the biosynthesis of LAM in M. smegmatis. The full HSQC spectra are available in the Supporting Information (Supporting Figure 1). Further chemical characterization, such as gas chromatography analysis, could provide accurate glycosyl composition of the truncated LAM; however due to the nature of the conditional mutant and a modest yield of cell mass generated, such analysis has proved to be extremely difficult to perform.

Analysis of Toll-like Receptor 2 Activation and Cytokine Production by Truncated LAM. Both LM and LAM display immunoregulatory and anti-inflammatory properties that affect the host immune response. A family of pattern recognition receptors, named TLR2 in association with TLR1 and TLR6, detect a wide range of ligands including mycobacterial LAM. Studies have demonstrated that the lipidic part of the molecule is required for its activity, whereas the glycosidic moiety was shown to contribute by directly controlling the extent of this activity. Thus, we explored the capacity of truncated LAM extracted from the conditional M. smegmatis ΔaftB::pMV306-afIB mutant depleted of AftB to induce TLR2-dependent innate immune responses. Previous studies demonstrated that a positive relation exists between the length of the mannan chain and the ability of the lipoglycan to activate TLR2. Lipoglycans with accessible long mannan domains, such as LM, were shown to be potent inducers of TLR2, whereas LAM molecules were poor activators of the TLR2 signaling due to their large arabinan domain somehow obstructing the availability of the mannan chain. Crude lipoglycan preparations extracted from M. smegmatis ΔaftB::pMV306-afIB, cultured in the presence and absence of acetamide, were subsequently subjected to hydrophobic and size exclusion chromatography to yield pure LAM fractions (Figure 5a,b). Earlier studies have reported that some of the LAM preparation from M. smegmatis may have been contaminated with lipopeptides and as a result had an immune stimulatory activity. Therefore, we have assessed both purified truncated and full-length LAM fractions for their activity on HEK293 cells that stably express human TLR4, which is the receptor for bacterial lipopolysaccharide (LPS) and lipid A. Cells were incubated with LPS, which served as a positive control, at concentrations ranging from 0.01 ng mL–1 to 10 μg mL–1 and both LAM fractions at concentrations ranging from 0.5 ng mL–1 to 10 μg mL–1. In the presence of

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Expanded region (δ 1H: 4.80–5.30, δ 13C: 100–112) of the two-dimensional 1H-13C HSQC spectra in D2O at 313 K of M. smegmatis LAM extracted from the aftB conditional mutant in the presence (a) and absence (b) of AftB.
LPS, a dose-dependent effect was observed, whereas HEK293-TLR4 cells stimulated with the purified full-length and truncated LAM resulted in similar results to those of the noninduced cells (Figure 6a). Therefore, we have concluded that the isolated LAM fractions were devoid of lipopeptide contamination. Subsequently, a derivative of HEK293 cells that stably expresses human TLR2 along with a NF-κB-inducible reporter system were incubated with full-length and truncated LAM extracted from M. smegmatisΔaftB pMV306-aftB grown in the presence and absence of acetamide, respectively, at concentrations ranging from 0.5 ng mL\(^{-1}\) to 10 μg mL\(^{-1}\). The synthetic Pam\(_3\)CSK\(_4\) lipopeptide was used as a positive control for TLR2 activation at concentrations ranging from 0.01 ng mL\(^{-1}\) to 1 μg mL\(^{-1}\). As shown in Figure 6b, both purified LAM molecules induced NF-κB activation in a dose-dependent fashion with the truncated LAM exhibiting a stronger TLR2 activation than compared to the full length LAM. A similar trend was observed in THP-1 cells (Figure 6d). Cells were preincubated with the anti-TLR2 or isotype control antibodies (10 μg mL\(^{-1}\)) for 30 min at 37 °C followed by incubation with full length LAM (5 μg mL\(^{-1}\)), truncated LAM (5 μg mL\(^{-1}\)), or Pam\(_3\)CSK\(_4\) lipopeptide (1 μg mL\(^{-1}\)). Both full length and truncated LAM induced activation of NF-κB (Figure 6e) and as a result production of IL-8 (Figure 6f) that was almost completely abolished by an anti-TLR2 antibody, thus demonstrating signaling through the TLR2.

A substantial number of biological activities have been associated with phosphatidylinositol-based glycolipids, notably LM and LAM.\(^{22,23,30}\) LM, a precursor of LAM, is composed of the conserved mannosyl-phosphate inositol anchor and mannan core. It was demonstrated to carry a dual modulatory function where it acts as a strong agonist for TLR2-dependent stimulation as well as exhibits TLR2 independent inhibition of cell activation and proinflammatory cytokine synthesis in murine primary macrophages.\(^{24}\) Further studies revealed that the degree of LM acylation plays an important role in pro- and anti-inflammatory properties\(^{21,32}\) whereas its mannan chain directly determines the lipoglycan activity.\(^{24}\) Specifically, the activity of lipoglycans seemed to increase with the number of Manp units composing the mannan core. Interestingly, mycobacterial LAMs have been shown to be poor agonists of TLR2 despite containing the LM glycosidic moiety. It is unclear how, but the large arabinan domain in LAM perturbs the biological activity of the mannan core. This was further confirmed by the chemical degradation of the arabinan domain of LAM and the regain of proinflammatory properties.\(^{26}\) The data presented here are in agreement with these findings as the truncated LAM isolated from the conditional aftB mutant demonstrated an increase in TLR2-dependent proinflammatory activity compared to the full-length LAM. It was suggested that the arabinan domain may exert its inhibitory effect by steric hindrance, which prevents interaction between TLR2 and the underlying mannan domain.\(^{26}\) The arrangement of the arabinofuranose residues in LAM is largely dependent on the flexibility of the glycosidic linkages and puckering of the rings. It is known that ring puckering along with the more rigid \(\beta(1 \rightarrow 2)\) linkages promote water mediated hydrogen bonding between different arabinofuranose residues. A structural study using fragments of the nonreducing termini of LAM and a murine antibody CS-35Fab has suggested that such hydrogen bonding between the arabinofuranose rings might contribute to the stabilization and orientation of the arabinan component of LAM.\(^{33}\) Therefore, we hypothesize that removal of the terminal \(\beta(1 \rightarrow 2)\) linkages can lead to a decrease in the propensity of hydrogen bonding and subsequently to a less organized and structurally compact arabinan domain as well as offer additional flexibility. This in turn can result in a more exposed “bioactive” mannan core. Further investigation is required to fully understand arabinan’s role of LAM in relation to TLR2 activation.\(^{26}\)

**METHODS**

**Plasmid Construction.** The 1938 bp coding region aftB (MSMEG_6400) was amplified by PCR from M. smegmatis mc\(^{3}\)SS
genomic DNA using the primer pair (restriction sites underlined): 5′-GAT CGA TCG GAT CCG TGC GCA TCA GCC TGT GGC-3′ and 5′-GAT CGA TCA TCG ATC TAC GGT CCC GTT GCC GGC-3′. A single copy integrating plasmid pMV306-aftB was generated by ligating a 2.6 kb inducible acetamide promoter digested with XbaI-BamHI and a 1.9 kb aftB digested with BamHI-ClaI into pMV306 digested with XbaI-ClaI resulting in a pMV306 construct containing aftB gene cloned downstream of the acetamide promoter. The merodiploid strain was generated by electroporation of M. smegmatis with pMV306-aftB and selecting for kanamycin resistant colonies.

Generation of ΔaftB Conditional Mutant in M. smegmatis. Approximately 1 kb upstream and downstream, flanking sequences of MSMEG_6400 (aftB) were PCR amplified from M. smegmatis mc2155 genomic DNA using the primer pairs: MS6400LL (5′-TTT TTT TTC CAT AAA TTG GGA GTT ACA CCA GCA GCT ACC-3′) and MS6400LR (5′-TTT TTT TTC CAT TTC TGG GAC CAG CAC ACC ATC ATC C-3′) as well as MS6400RR (5′-TTT TTT TTC CAT CTT TGG GAC CAG CAC ACC ATC ATC C-3′) as well as MS6400RL (5′-TTT TTT TTC CAT AGA TTT GTG GAT GGG CAT GCT GGG CAT GAA CG-3′) and MSMEG6400RR (5′-TTT TTT TTC CAT CTT TGG GAC CAG CAC ACC ATC ATC C-3′). Following restriction digestion of the primer incorporated Van91I sites, the PCR fragments were cloned into Van91I-digested p0004S to yield the plasmid pΔMSMEG6400. The resultant plasmid was then packaged into the temperature sensitive phage phAE159 as described previously to yield the knockout phage phΔMSMEG6400. Specialized transduction was performed as described earlier, except that the host strain was the merodiploid strain and half of the transduction mix was spread on TSB agar plates containing kanamycin, hygromycin, and 0.2% (w/v) acetamide while the other half was spread on plates lacking acetamide. Deletion of the aftB gene was confirmed using Southern blot analysis.

Conditional Depletion of AftB. Strains to be tested were grown in either TSB or minimal medium supplemented with 0.05% (v/v) Tween-80 and 0.2% (w/v) acetamide to an OD 600 nm of 0.5. Cells were washed twice with media to remove traces of acetamide and resuspended in the original volume of appropriate media. Culture was used as a 20% inoculum in minimal media and grown for 12 h to deplete intracellular AftB. The depleted subculture served as inoculum (5%) for cultures with or without 0.2% (w/v) acetamide. Aliquots were taken and labeled with either [1, 2-14C]-glucose 1 μCi mL−1 or [13C6]-glucose for 10 h. Cells were harvested, washed with phosphate buffered saline, and dried.

Cell Stimulation Assays. For stimulation assays, HEK293 and THP-1 cells were plated at 50 000 cells per well. Cells were stimulated with LAM at 0.5 ng µL−1 to 10 µg µL−1 in a final volume of 200 µL. Nonstimulated cells and cells stimulated with Pam3CSK4 (InvivoGen) or LPS (InvivoGen) served as negative and positive controls, respectively. Alkaline phosphatase activity was measured after 16−18 h by mixing 20 µL of the culture supernatant and 180 µL of QuantiBlueTM, reading absorbance at OD 630 nm. To investigate TLR2 dependence, THP-1 cells were preincubated for 30 min at 37 °C with 5 or 10 µg µL−1 of anti-TLR2 monoclonal antibody (clone T2S,
Cytokine ELISA. Culture supernatants from THP-1 cells were harvested and assayed for IL-8 cytokine production using commercially available ELISA kit and according to the manufacturer’s instructions (Ready Set Go ELISA kits, eBioscience).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00898.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +44 (0)121 41 58125. Fax: +44 (0)121 41 45925. E-mail: g.besra@bham.ac.uk.

ORCID

Gurdyal S. Besra: 0000-0002-5605-0395

Notes

The authors declare no competing financial interest.

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