Tsukamurella paurometabola Lipoglycan, a New Lipoarabinomannan Variant with Pro-inflammatory Activity*

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The genus Tsukamurella is a member of the phylogenetic group nocardioform actinomycetes and is closely related to the genus Mycobacterium. The mycobacterial cell envelope contains lipoglycans, and of particular interest is lipoarabinomannan, one of the most potent mycobacterial immunomodulatory molecules. We have investigated the presence of lipoglycans in Tsukamurella paurometabola and report here the isolation and structural characterization of a new lipoarabinomannan variant, designated TpaLAM. Matrix-assisted laser desorption ionization-mass spectrometry analysis revealed that TpaLAM had an average molecular mass of 12.5 kDa and consequently was slightly smaller than Mycobacterium tuberculosis lipoarabinomannan. Using a range of chemical degradations, NMR experiments, capillary electrophoresis, and mass spectrometry analyses, TpaLAM revealed an original carbohydrate structure. Indeed, TpaLAM contained a mannosylphosphatidylmyo-inositol (MPI) anchor glycosylated by a linear (α1→6)-Man mannop albumin domain, which is further substituted by an (α1→5)-Araf chain. Half of the Ara f units are further substituted at the O-2 position by a Manp (α1→2)-Manp-(α1→3) dimannoside motif. Altogether, TpaLAM appears to be the most elaborated non-mycobacterial LAM molecule identified to date. TpaLAM was found to induce the pro-inflammatory cytokine tumor necrosis factor (TNF-α) when tested with either human or murine monocyte/macrophage cell lines. This induction was completely abrogated in the presence of an anti-toll-like receptor-2 (TLR-2) antibody, suggesting that TLR-2 participates in the mediation of TNF-α production in response to TpaLAM. Moreover, we established that the lipomannan core of TpaLAM is the primary moiety responsible for the observed TNF-α-inducing activity. This conclusively demonstrates that a linear (α1→6)-Man chain, linked to the MPI anchor, is sufficient in providing pro-inflammatory activity.

There is a bewildering range of aerobic actinomycetes, found in almost any environment imaginable, with some pathogenic to humans and others that are not (1). The aerobic actinomycetes may be further subdivided into the “ncardioform actinomycetes” (2). This informal terminology is now widely used to describe a number of organisms with similar characteristics, with key members including mycobacteria, nocardia, rhodococcus, and corynebacteria (3). Unlike the previously mentioned members the genus Tsukamurella is in its infancy, whereas the type strain, Tsukamurella paurometabola, has had a long taxonomic history, with names including Corynebacterium paurrometabolum (4), Gordona aurantiaca (5), and Rhodococcus aurantiacus (6). This taxonomical puzzle was finally resolved, when in 1988 Collins et al. (7) showed that the 16S RNAs of R. aurantiacus and C. paurrometabolum were 99% homologous. And so they proposed reclassifying and renaming this organism T. paurometabola, after the microbiologist M. Tsukamura, who first isolated the species (7).

Cases of human infection with T. paurometabola are infrequent, nevertheless diagnosis rates are increasing, typically in patients with underlying predisposing factors, including immunosuppression (8, 9), chronic pathology (6), and indwelling foreign bodies (10). However, there are a number of reported cases in which infected patients had no underlying factors, with Grael et al. (11) describing an inflammatory cutaneous infection in an otherwise healthy individual.

All members of the nocardioform actinomycetes possess a similar whole cell carbohydrate composition, whereas the majority also contain long-chain branched fatty acids, termed mycolic acids (12). The majority of our current knowledge about actinomycetes cell wall architecture comes from pioneering studies on mycobacterial strains (13). Such work led to the identification, within the cell envelope, of a biosynthetically related family of glycolipids, phosphatidyl-xyo-inositol mannosides (PIMs),1 and lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM) (13). Mycobacterial LAM is a large heterogeneous macroamphiphile that possesses three distinct

1 The abbreviations used are: PIM, phosphatidyl-xyo-inositol mannoside; Ara f, arabinofuranose; APTS, 1-aminopyrene-3,6,8-trisulfonate; AsuLAM, gynocolatosis sulphurea lipogranibonemannan; αTpaLAM, α-mannosidase-treated TpaLAM; CE-LIF, capillary electrophoresis-laser-induced fluorescence; CE/ESI, capillary electrophoresis/electrospray ionization; dTpaLAM, deacylated TpaLAM; GC, gas chromatography; HMBC, heteronuclear multiple bond correlation spectroscopy; HMQ, heteronuclear multiple quantum correlation spectroscopy; HOHH, homonuclear Hartmann-Hahn spectroscopy; IL, interleukin; LM, lipoarabinomannan; LAM, lipomannan; MahTpaLAM, mild acidic-hydrolyzed TpaLAM; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Manp, mannosylpyranose; ManLAM, LAM with mannosyl caps; MPI, mannosylphosphatidyl-xyo-inositol; MS, mass spectrometry; PILAM, LAM with phosphoglycoside caps; ReqLAM, rhodococcus ggs lipoarabinomannan; t, terminal; TLR, toll-like receptor; TNF-α, tumor necrosis factor α; TpaLAM, Tsukamurella paurometabola lipogranibonemannan.
domains, a mannosylphosphatidylyl-myo-inositol (MPI) anchor, a carbohydrate backbone, and various capping motifs (14, 15). The carbohydrate backbone is composed of two homopolysaccharides, D-mannan and D-arabian. In all species described to date the D-mannan domain exists as a linear α(1→6)-Man backbone substituted according to the species at the O-2 or O-3 positions by single Manp residues. The D-arabian domain consists of a linear α(1→5)-Ara p backbone punctuated by branching fashioned from 3,5-O-linked α-Ara p residues (14, 15).

In slow growing mycobacteria, such as Mycobacterium tuberculosis, the capping motifs consist of Manp to inhibit the production of pro-inflammatory cytokines, such as TNF-α and IL-12 (16, 17); whereas fast growing mycobacteria, such as Mycobacterium smegmatis, possess phosphoinositol (18, 19), resulting in LAM being termed either ManLAM or PILAM, respectively. Indeed, any capping motifs (20), termed AraLAM. These subtle differences in LAM structures to that of mycobacterial LAM. In particular, several studies have demonstrated that a LAM-like molecule from T. paurometabola, type strain T. paurometabola, DSM 20162, was purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Germany). It was routinely grown at 30 °C in GYM streptomycin medium, which contained 4 g of glucose, 4 g of yeast, and 10 g of maltose extract per liter of deionized water supplemented with 0.05% (w/v) Tween 80. Cells were grown to late log phase, harvested by centrifugation, washed, and lyophilized.

We report here the isolation and structural characterization of a lipoglycan originating from T. paurometabola. Furthermore, we provide evidence for the molecular motifs underlying bacterial lipoglycan mediated pro-inflammatory cytokine responses.

**Materials and Methods**

**Bacteria and Growth Conditions**—T. paurometabola, type strain DSM 20162, was purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Germany. It was routinely grown at 30 °C in GYM streptomycin medium, which contained 4 g of glucose, 4 g of yeast, and 10 g of maltose extract per liter of deionized water supplemented with 0.05% (w/v) Tween 80. Cells were grown to late log phase, harvested by centrifugation, washed, and lyophilized.

**Purification of TpaLAM**—Purification procedures were adapted from protocols established for the extraction and purification of mycobacterial lipoglycans (31, 32). Briefly, the cells were delipidated at 60 °C by mixing in CHCl3/CH3OH (1:1, v/v) overnight. The organic extract was removed by filtration, and the delipidated biomass was resuspended in deionized water and disrupted by sonication (MSE Soniprep, 12 micro amplitudes, 60 s on, 90 s off for 10 cycles, on ice). The cellular glycans and lipoglycans were further extracted by refluxing the broken cells in 50% ethanol at 65 °C overnight. Contaminating proteins and glucans were removed by enzymatic degradation using proteinase K and α-mannosidase, respectively, treatments followed by dialysis. The resulting extract was resuspended in buffer A, 15% propan-1-ol in 50 mM ammonium acetate, and loaded onto an octyl-Sepharose CL-4B column (50 × 2.5 cm) and eluted with 400 ml of buffer A at 5 ml/h, enabling the removal of non-lipidic moieties (22). The retained lipoglycans were eluted with 400 ml of buffer B, 50% propan-1-ol in 50 mM ammonium acetate. The resulting lipoglycans were resuspended in buffer C, 0.2 mM NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA, and 10 mM Tris, pH 8, to a final concentration of 200 mg/ml and loaded onto a Sephacryl S-200 HR column (50 × 2.5 cm) and eluted with buffer C at a flow rate of 5 ml/h. Fractions (1.25 ml) were collected and analyzed by SDS-PAGE followed by periodic acid-silver nitrate staining. The resulting lipoglycan fractions were pooled, dialyzed extensively against water, lyophilized, and stored at −20 °C.

**Preparation of Chemically or Enzymatically Modified TpaLAM**—Deacylated TpaLAM (dTpaLAM) was obtained by incubating 100 μg of TpaLAM with 200 μl of 0.1 NaOH for 2 h at 37 °C. The reaction was stopped by extensive dialysis against water. TpaLAM lipomannan core (i.e., mild acid hydrolyzed TpaLAM, mTpaLAM) was prepared by mild acid hydrolysis (0.1 M HCl at 110 °C for 25 min) of TpaLAM. TpaLAM lipomannan core was recovered after dialysis against water and analyzed for carbohydrate content via capillary electrophoresis coupled to laser-induced fluorescence (CE-LIF) as described below. α-Exomannosidase-treated TpaLAM (αTpaLAM) was prepared by incubating TpaLAM (100 μg) for 6 h at 37 °C in 50 μl of a jack bean α-mannosidase (Sigma) solution (2 mg/ml, 0.1 mM sodium acetate buffer, pH 4.5, 1 mM ZnSO4). After a second addition of 50 μl of enzyme solution, the reaction was continued overnight. The reaction products were then dialyzed against 50 mM NH4HCO3, pH 7.6. Elimination of α-mannosidase was achieved by denaturation (2 min at 110 °C) followed by overnight trypsin digestion (37 °C, 3.2 μg of trypsin). αTpaLAM was recovered after dialysis against water and analyzed for cap contents by CE-LIF (22).

**MALDI-TOFMS**—The matrix was used 2,5-dihydroxybenzoic acid at a concentration of 10 μg/ml in a mixture of water/ethanol (1:1, v/v). 0.5 μl of TpaLAM, at a concentration of 10 μg/ml, was mixed with 0.5 μl of the matrix solution. Analyses were performed on a Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using linear mode detection. Mass spectra were recorded in the negative mode using a 300-ms time delay with a grid voltage of 94% of full accelerating voltage (20 kV) and a guide wire voltage of 0.1%. The mass spectra were mass assigned using external calibration.

**Fatty Acid Analysis**—200 μg of TpaLAM was deacylated using strong alkaline hydrolysis (200 μl of 1 N NaOH at 110 °C for 2 h). The reaction mixture was neutralized with HCl, and the liberated fatty acids were extracted three times with chloroform and, after drying under nitrogen, were methylated using three drops of 10% (w/w) BF3 in methanol (Fluka) at 60 °C for 5 min. The reaction was stopped by the addition of water and the fatty acid methyl esters were extracted three times with chloroform. After drying under nitrogen, the fatty acid methyl esters were eluted in 10 μl of pyridine and trimethylsilylated using 10 μl of hexamethyldisilazane and 5 μl of trimethylchlorosilane at room temperature for 15 min. After drying under a stream of nitrogen, the fatty acid derivatives were solubilized in cyclohexane before analysis by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

**Glycosidic Linkage Analysis**—Glycosyl linkage composition was performed according to the modified procedure of Ciucanu and Kerek (33). The per-O-methylated TpaLAM was hydrolyzed using 500 μl of 2 M trifluoroacetic acid at 110 °C for 2 h, reduced using 350 μl of a 1 M NaOH solution of NaBD3, (1 M NH4OH/H2O, 1:1, v/v) and per-O-acetylated using 300 μl of acetic anhydride for 1 h at 110 °C. The resulting alditol acetates were solubilized in cyclohexane before analysis by GC and GC/MS.

**APTS Derivatization**—1–5 μl of lipoglycans, in the presence of mannoheptose as an internal standard, were hydrolyzed using either strong acid hydrolysis (30 μl of 2 M trifluoroacetic acid at 110 °C for 2 h) or total carbohydrate analysis or mild acid hydrolysis (30 μl of 0.1 M HCl at 110 °C for 20 or 30 min) (caps analysis). The samples were dried and mixed with 0.3 μl of 0.2 M 1-aminoopyrene-3,6,8-trisulfonate (APTS) (Interchem, Montluçon, France) in 15% acetic acid and 0.3 μl of a 1 M sodium cyanoborohydride solution dissolved in tetrahydrofuran. The reaction mixture was heated at 55 °C for 90 min and subsequently quenched by the addition of 20 μl of water. A 2 μl solution of the APTS solution was orginated on a 10-fold dilution before being subjected to capillary electrophoresis.

**CE-LIF**—Analyses were performed on a PACE capillary electrophoresis system (Beckman Instruments, Inc.) with the cathode on the injection side and the anode on the detection side (reverse polarity). The electropherograms were acquired and stored on a Dell XPS P60 computer using the software package (Beckman Instruments, Inc.). APTS derivatives were loaded by applying 0.5 p.s.i. (3.45 kPa) vacuum for 5 s (6.5 ml injected). Separations were performed using an uncoated fused-silica capillary column (Sigma, Division Supelco, Saint-Quentin-Fallavier, France) of 50-μm internal diameter with 40 cm of effective length (47 cm total length). Analyses were carried out at a temperature of 25 °C with an applied voltage of 20 kV. Buffer consisted of 1% (w/v), triethylamine 30 mM in water, pH 3.5, as running electrolyte. The detection system consisted of a Beckman laser-induced fluorescence (LIF) equipped with a 4-milliwatt argon-ion laser with the excitation wavelength of 488 nm and emission wavelength filter of 520 nm.
A lipoglycan with a SDS-PAGE migration similar to that of *M. tuberculosis* LAM was purified from *T. paurometabola* (Fig. 1A). The negative MALDI mass spectrum of the lipoglycan exhibited a broad unresolved peak centered at m/z 12500, indicating a molecular mass around 12.5 kDa for the major molecular species of this lipoglycan (Fig. 1B). CE-LIF analysis of the total acid hydrolyzed lipoglycan showed that it contained Ara and Man, in a ratio of 1:1.7. In addition, myo-inositol, glycerol, and fatty acids were also detected by GC analysis. The predominant fatty acids identified were palmitic (C16:0, 50%) and octadecenoic (C18:1, 15%) acids. Altogether, the lipoglycan exhibited five anomeric signals at δ 4.85 (III 1), δ 4.94 (IV 1, GB), and δ 5.05 (V 1) and was subsequently termed TpaLAM.

RESULTS

General Features

A lipoglycan with a SDS-PAGE migration similar to that of *M. tuberculosis* LAM was purified from *T. paurometabola* (Fig. 1A). The negative MALDI mass spectrum of the lipoglycan exhibited a broad unresolved peak centered at m/z 12500, indicating a molecular mass around 12.5 kDa for the major molecular species of this lipoglycan (Fig. 1B). CE-LIF analysis of the total acid hydrolyzed lipoglycan showed that it contained Ara and Man, in a ratio of 1:1.7. In addition, myo-inositol, glycerol, and fatty acids were also detected by GC analysis. The predominant fatty acids identified were palmitic (C16:0, 50%) and octadecenoic (C18:1, 15%), with smaller amounts of stearic (C18:0, 15%) and tuberculostearic (methyl-10-methyloctadecanoic, C19:0, 15%) acids. Altogether, the lipoglycan exhibited five anomeric signals at δ 4.85 (III 1), δ 4.94 (IV 1, GB), and δ 5.05 (V 1) and was subsequently termed TpaLAM.

Structural Characterization

NMR Signal Assignment—Per-O-methylation analysis of TpaLAM indicated the presence of 5-Araf, t-Manp, 2,5-Araf, and 2-Manp residues in similar ratios, with slightly less 6-Manp detectable. Accordingly, the 1H-NMR anomeric region of TpaLAM exhibited five anomeric signals at δ 5.07 (I 1), δ 5.02 (II 1), δ 4.94 (III 1), δ 4.85 (IV 1), and δ 4.71 (V 1), in a ratio 1.4/1.5/1.8/1.5/1 (Fig. 2A). As revealed by the 1H-13C HMBC spectrum, their corresponding anomeric carbons resonate at δ 30.27 and δ 33.18.
and IV) was based on their C-1 chemical shift at δ 103.1 (III), δ 109.3 (IV), and δ 100.7 (V), respectively (Fig. 2D). Proton and carbon resonances of the different spin systems were assigned from 1H-13C HMQC and 1H-1H HOHAHA experiments (partially shown in Figs. 2C, 2D, and Fig. 3, respectively) and were based on our previous studies with mycobacterial LAMs (17, 39) and LAM-related structures (25, 40, 41). The assignments are summarized in Table I. Spin systems I, II, III, IV, and V were unambiguously assigned to 2-α-Manp, 2,5-α-Araf, t-α-Manp, 5-α-Araf, and 6-α-Manp, respectively, according to the following lines of evidence.

The α-anomeric configuration of 2,5-α-Araf and 5-α-Araf (II and IV) was based on their C-1 chemical shift at δ 107.1 and δ 109.3, respectively, compared with α/β-Araf units in mycobacterial LAM (α-Araf: δC-1 108–110; β-Araf: δC-1 103) (17, 18). Moreover, this was in agreement with the magnitude of the 2J_H1,C1 coupling constant determined as 1.9 Hz for spin system IV (α-Araf: 2J_H1,C1 1.7 Hz; β-Araf: 2J_H1,C1 4.6 Hz (42)) (Table I). The C-4 carbon chemical shift at δ 82.9 (II) and δ 83.0 (IV), respectively (Table I and Fig. 2C) confirmed a furanose ring form (α-Araf: δC-4 84.9; α-Arap: δC-4 69.4 (43)). Glycosylation at position 5 was shown through the deshielding of the C-5 resonances at δ 68.1 (II) and IV (Table I and Fig. 2C) as compared with the unsubstituted t-α-Araf units present in LAM from Rhodococcus equi (RegLAM), with C-5 resonances at δ 64.2 (δ 3.9 ppm) (25). Glycosylation at position 2 of 2,5-α-Araf (II) was determined by the deshielding of its C-2 resonance at δ 88.1 (Table I and Fig. 2C) as compared with the C-2 resonance of 5-α-Araf (IV) at δ 96.3 (3α 5.2 ppm). Glycosylation at O-2 was in agreement with the shielding observed for the C-1 of 2,5-α-Araf (II) at δ 107.1 compared with C-1 to 5-α-Araf (IV) at δ 109.3, as previously reported (17). The α-anomeric configuration of Manp units was determined through the magnitude of their 3J_H1,C1 coupling constants determined as 172, 172, and 170 Hz for spin systems I, III, and V, respectively (α-O-Manp: 3J_H1,C1 170 Hz; β-O-Manp: 3J_H1,C1 161 Hz (44)) (Table I). Glycosylation at position 2 of 2-α-Manp (I) was determined by the deshielding of its C-2 resonance at δ 98.5 (I) (Table I and Fig. 2C) as compared with the C-2 resonance of t-α-Manp in M. tuberculosis LAM at δ 78.5 (I) (39). Spin systems III and V showed proton and carbon chemical shifts (Table I) typical of those of t-α-Manp and 6-α-Manp units found in mycobacterial LAM (39). Moreover, the pyranose ring of systems I and III was confirmed by the cross-peaks observed on the 1H-13C HMBC experiment (Fig. 2B) between their H-1 at δ 5.07 and δ 4.94, respectively, and their respective C-5 at δ 75.1 and 75.5, respectively.

Arabinan Domain and Mannooligosaccharide Caps—The sequence of the different units was investigated by 1H-13C HMBC....
NMR experimentation (Fig. 2B). H-1 of 2-α-Manp (I) at δ 5.07 showed intracyclic connectivities with their C-2 at δ 78.5, C-3 at δ 71.6, and C-5 at δ 75.1. An additional intercyclic connectivity with C-2 of 2,5-α-Araf (II) at δ 88.1 indicated that 2-α-Manp were linked at O-2 of 2,5-α-Araf. H-1 of 2-α-Manp (I) showed connectivities with their own C-3, C-4, and C-5 at δ 77.4, 82.9, and 68.1, respectively. Because it was confirmed that the 2,5-α-Ara units were in the furanose form, the cross-peak between the H-1 and C-5 resonances could only be assigned to an intercyclic connectivity, establishing that 2,5-α-Ara are interconnected by (1→5) linkages. Nevertheless, as C-5 resonances of 2,5-α-Araf and 5-α-Araf units cannot be distinguished (Fig. 2C and Table I), one cannot exclude the possibility of an interconnection of 2,5-α-Araf and 5-α-Araf units by an (α1→5) linkage. No correlation was observed between H-1 of t-α-Manp (III) and C-2 of 2,5-α-Araf (II) on the HMBC spectrum (Fig. 2B), indicating that single mannose units do not substitute the arabinan chain. Altogether the data

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a ND, not determined.
b The chemical shift of these protons could not be determined precisely and are given within an interval.
indicated that 2,5-α-Araf units were substituted at O-2 by the Manp-(α1→2)-Manp-(α1→) oligomannoside motif. Indeed, the correlation observed between H-1 of 2-O-Manp with their own C-2 could be due to intracyclic connectivity, intercyclic connectivity, or both. Consequently, an interconnection of 2-α-Manp by an (α1→2) linkage cannot be excluded from NMR data. Nevertheless, peak integration of the 1D 1H spectrum showed that t-α-Manp, 2-α-Manp, and 2,5-α-Araf are present in similar amounts, suggesting that the dimannoside, Manp-(α1→2)-Manp-(α1→), is the most abundant motif.

To investigate the degree of mannosylation present in the side chain caps, TpaLAM was partially hydrolyzed using mild acid conditions (0.1 M HCl for 20 min at 110 °C), APTS-derivatized, and subjected to CE-LIF analysis (45). The electropherogram (Fig. 4) exhibited, beside the peaks assigned to Ara-APTS (I), Man-APTS (II), and mannoheptose-APTS (III, internal standard), two main peaks (IV and V) that were likely to correspond to tri- and tetrasaccharide-APTS compounds, based on their retention times. But, compounds IV and V did not co-inject with the corresponding structural compounds obtained by mild acid hydrolysis of TpaLAM and V did not co-inject with the corresponding structural compounds, based on their retention times. But, compounds IV likely to correspond to tri- and tetrasaccharide-APTS compounds.

The relative abundance of 2,5-α-Araf, 2-α-Manp, and t-α-Manp units, determined by integration of the anomic proton signals, was in the ratio 1.5/1/4/18. 2,5-α-Araf units were linked to the dimannoside units, and consequently were unlikely to be substituted by lateral (α1→5)-Araf chains. Moreover, methylation analysis showed that the occurrence of lateral Araf chains, which were substituted by t-Ara units, is statistically very weak. Taken together, one may conclude that the arabinan domain is likely to be arranged as a linear (α1→5)-Araf chain,
The presence of 6-Man units, but almost no 2,6-Man domain was likely to consist of a linear (branching degree of 50% for the arabinan chain). This assumption is contrary to all other LAM-like molecules, the mannose moiety. One may explain the differences between mahTpaLAM and TpaLAM by reasoning that the mannan core in TpaLAM is possessed entirely from 6-mannose units.

Moreover, GC analyses (not shown) demonstrated that the mannan core fraction contained inositol and fatty acids as opposed to the mono- and oligosaccharide fraction. TpaLAM lipomannan core fraction contained inositol and fatty acid methyl esters. The occurrence of a product that afforded in CI mode (M+1) allowed us to positively identify this compound as 2,6-diacetyl-1,3,4,5-tetramethylinositol moiety. These results strongly suggest that TpaLAM possesses an MPI anchor characterized by a diglycosylated myoinositol unit substituted at positions 2 and 6, as established for mycobacterial LAM (14, 47, 48). This was further supported by the identification of phosphatidyl-myoinositol dimannosides (PIM₂) species, considered to be LAM precursors, within T. paurometabola lipidic fractions (data not shown). Moreover, 1D ³¹P NMR analysis (15, 40) of TpaLAM (not shown) indicated the occurrence of MPI anchor acyl-forms bearing essentially diacylglycerol units.

**FIG. 5.** CE-LIF analysis (A) and 1D ¹H NMR spectrum in MeSO-d6 at 343K (B) of TpaLAM lipomannan core. A, native TpaLAM (upper electropherogram) or TpaLAM lipomannan core (lower electropherogram) in the presence of mannoheptose as internal standard were hydrolyzed with 2% trifluoroacetic acid at 110°C for 2 h, APTS-derivatized, and submitted to CE-LIF migration (same conditions as in Fig. 4A). I, Ara-APTS; II, Man-APTS; III, internal standard, mannoheptose-APTS. B, expanded regions (δH: 4.62–5.15) of 1D ¹H NMR spectra of native TpaLAM (upper spectrum) or TpaLAM lipomannan core (lower spectrum) are shown. I, 2-α-Manp; II, 2,5-α-Araf; III, t-α-Manp; IV, 5-α-Araf; and V, 6-α-Manp.

with half of the units being substituted at O-2 by the oligomannoside motifs. Indeed, integration of the anomeric proton resonances at δH 4.6–5.15 was dominated by a single resonance at δH 4.92, indicating a branching degree of 50% for the arabian chain.

**Mannan Domain**—Per-O-methylation analysis indicated the presence of 6-Manp units, but almost no 2,6-Manp units were detected in TpaLAM, indicating that the TpaLAM mannann domain was likely to consist of a linear (α1–6)-Man polymer. This assumption is contrary to all other LAM-like molecules analyzed so far, in that these mannan domains are composed from branched chains. To confirm this assumption, TpaLAM lipomannan core was obtained after mild acid hydrolysis and dialysis to separate the released mono- and oligosaccharides (those previously analyzed by CE in Fig. 4) from the non-hydrolyzed lipomannan core (41). CE-LIF (Fig. 5A) and GC (not shown) analyses of the totally hydrolyzed recovered lipomannan core fraction showed that it contained essentially mannosyl residues, as compared with the native TpaLAM molecule. In addition, the anomeric zone of the 1D ¹H NMR spectrum of TpaLAM lipomannan core (Fig. 5B) was dominated by a single resonance at δH 4.71 attributed to the H-1 of the 6-α-Manp units. Moreover, GC analyses (not shown) demonstrated that the TpaLAM lipomannan core fraction contained inositol and fatty acids as opposed to the mono- and oligosaccharide fraction. Taken together, these data demonstrate that, in contrast to mycobacterial LAM and other LAM-like molecules, the mannan domain in TpaLAM is a linear non-branching chain composed entirely from 6-α-Manp residues; however, similarly to mycobacterial LAM and other LAM-like molecules, the mannan domain is linked to the MPI anchor.

**MPI Anchor**—Per-O-methylation analysis showed the presence of a product that afforded in CI mode (M+H)⁺ and (M+NH₄)⁺ ions at m/z 321 and 338, respectively, characteristic of a di-acetylated, tetra-methylated inositol (46). EI/MS fragments at m/z 200, 191, and 75 allowed us to positively identify this compound as 2,6-diacytetyl-1,3,4,5-tetramethylinositol moiety (20, 46). These results strongly suggest that TpaLAM activity is predominantly mediated by its lipomannan moiety. One may explain the differences between mahTpaLAM and TpaLAM by reasoning that the mannan core in TpaLAM is likely to be partially veiled by the arabian chain, leading to a reduced activity for native TpaLAM, when compared with the free lipomannan moiety.

**DISCUSSION**

To date a number of LAM variants have been isolated and characterized from a number of non-mycobacterial strains (24–30). These variants, although different in overall fine structure, exhibit a macro-structure in common with, but distinctly dif-
tested at 10 (THP-1 (and B) was tested at 0.2
TNF-
Polymyxin B, when previously added, had no effect on the amount of /H9262 of 0.2
compared with mycobacterial LAM, a fact evidenced not only TpaLAM exhibits the most similar architectural features when characterized the LAM from different from mycobacterial LAM. In the present report we have hydrolyzed TpaLAM.
TpaLAM; dTpaLAM, deacylated TpaLAM; mahTpaLAM, mild acidic-
FIG.6 .
FIG.7 .
Structural model of TpaLAM. TpaLAM is comprised from a linear (α1→6)-mannan domain linked to a MPI anchor and a (α1→5)-Araf domain that is substituted at half of the O-2 positions by the Manp-(α1→2)-Manp-(α1→) dimannoside motif. From the molecular mass of 12.5 kDa and integration of anomic protons signals, m, n, and p can be estimated at 11, 16, and 16, respectively. The proposed model does not account for the relative distribution of the substituted and non-substituted 5-Araf units.
by their high molecular mass, with MALDI/MS providing an average molecular mass centered around 12.5 kDa but with their almost identical migration on SDS-PAGE when compared with mycobacterial LAM. The observation that TpaLAM is alike to mycobacterial LAM is interesting as Tsukamurella sp. are the most closely related species to mycobacteria (51), which corresponds with the notion that lipoglycan composition may be of some chemotaxonomic use (28).
TpaLAM has a similar carbohydrate composition to mycobacterial LAM, with an Ara:Man ratio being ~1:1.7. Per-O-methylation and detailed NMR studies revealed that TpaLAM was comprised from mainly linear (α1→6)-mannan and (α1→5)-Araf domains. The linear polymer of (α1→6)-Manp units lack any side chains, which is in complete contrast to mycobacterial LAM that exhibits a highly branched mannan domain (15). In addition, the arabinan domain is characterized by the presence of 2,5-α-Araf units, which were first identified in LAM from Amycolatopsis sulphurea (27). These branched Araf units differ from those found in mycobacterial LAM, where branching is fashioned from 3,5-α-Araf units. TpaLAM possessed a sophisticated arabinan domain, composed of a single (α1→5)-Araf chain, as opposed to the arabinan domain found in mycobacterial LAM, which is composed from a linear (α1→5)-Araf chain to which lateral side chains are attached. The lack of side chains in TpaLAM presumably arises from the fact that T. paurometabola lacks the relevant arabinosyltransferases found in mycobacterial strains. In addition, ~50% of these units were further substituted at the O-2 position by manno-oligosaccharide units, characterized by the Manp-(α1→2)-Manp-(α1→) dimannoside motif, depicted in Fig. 7.
The capping of the arabinan chain by manno-oligosaccharide units directly onto the O-2 of the α-Araf chain is a further dissimilarity to that of mycobacterial LAM, where the manno-oligosaccharide caps are located at the non-reducing end of lateral arabinan chains. 1H-13C HMBC NMR experiments revealed that the 2,5-α-Araf units were substituted at O-2 by the Manp-(α1→2)-Manp-(α1→) dimannoside motif, but the presence of longer α(1→2) oligosaccharide side chains could not be excluded due to the fact that the correlation observed between H-1 of 2-α-Manp with their own C-2 could be due to both intra- and intercyclic connectivities. Nevertheless, knowing that t-A-Manp units were present only in the caps, the similar intensity

Different from mycobacterial LAM. In the present report we have characterized the LAM from T. paurometabola. To date, TpaLAM exhibits the most similar architectural features when compared with mycobacterial LAM, a fact evidenced not only

by

their

high

molecular

mass,
of the $^1$H anomic signals of t-α-Manp, 2-α-Manp, and 2,5-α-Araf suggested the dimannoside as the most abundant motif. This was further confirmed by CE-LIF and CE/ESI-MS analyses on mild acid-hydrolyzed TpaLAM that showed Man-Manara as the main reaction product (90%), with a very small amount of Man-Man-Ara (10%) detectable.

1D $^3$P NMR analysis of TpaLAM showed that the MPI anchor contained essentially di-acylglycerol acyl forms. Furthermore, we identified 2,6-diacyt-1,3,4,5-tetramethylinositol during per-O-methylation analysis, indicative of a MPI anchor characterized by a diglycosylated myo-inositol unit substituted at positions 0-2 and 0-6 (20, 46).

In summary, TpaLAM exhibits an original structure with the same core domains as described for mycobacterial LAM, but with some subtle differences, which explains the differences found in the relative molecular masses of TpaLAM and ManLAM. Indeed, lacking in TpaLAM, are first, the existence of several lateral arabinan chains found substituting the arabinan backbone, and second, the branching units of the mannan core. Nevertheless, at this time, we appear to have the most elaborated non-mycobacterial LAM molecule identified to date. The structural model proposed for TpaLAM is detailed in Fig. 7.

A current paradigm in mycobacteriology is that LAM molecules have immunomodulatory roles, conceivably during the pathogenesis of tuberculosis (14, 15, 47). More explicitly, ManLAMs have the ability to inhibit the production of inflammatory cytokines such as TNF-α and interleukin-12 (21, 22), whereas PILAMs are able to stimulate such cytokines, via a TLR-2-dependent signaling cascade in macrophages (18, 23, 24). Moreover, it has been recently demonstrated that most ManLAMs are able to stimulate such cytokines, via a TLR-3-dependent signaling pathway (19, 25). In contrast, TpaLAM has been shown to induce TNF-α production in a TLR-2-dependent fashion, as previously reported for the mycobacterial manno-conjugates, PIMs (49) and LMs (50).

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