Truncated Structural Variants of Lipoarabinomannan in Ethambutol Drug-resistant Strains of Mycobacterium smegmatis

INHIBITION OF ARABINAN BIOSYNTHESIS BY ETHAMBUTOL*

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The anti-tuberculosis drug, ethambutol (Emb), was previously shown to inhibit the synthesis of arabinans of both the cell wall arabino-galactan (AG) and lipoarabinomannan (LAM) of Mycobacterium tuberculosis and other mycobacteria. However, an Emb-resistant mutant, isolated by consecutive passage of the Mycobacterium smegmatis parent strain in media containing increasing concentrations of Emb, while synthesizing a normal version of AG, produced truncated forms of LAM when maintained on 10 μg/ml Emb (Mikušová, K., Slayden, R. A., Besra, G. S., and Brennan, P. J. (1995) Antimicrob. Agents Chemother. 39, 2482–2489). We have now isolated and characterized the truncated LAMs made by both the resistant mutant and a recombinant strain transfected with a plasmid containing the emb region from Mycobacterium avium which encodes for Emb resistance. By chemical analysis, endoarabinanase digestion, high pH anion exchange chromatography, and mass spectrometry analyses, truncation was demonstrated as primarily a consequence of selective and partial inhibition of the synthesis of the linear arabinan terminal motif, which constitutes a substantial portion of the arabinan termini in LAM but not of AG. However, at higher concentrations, Emb also affected the general biosynthesis of arabinan destined for both AG and LAM, resulting in severely truncated LAM as well as AG with a reduced Ara:Gal ratio. The results suggested that Emb exerts its antitymococbial effect by inhibiting an array of arabinosyltransferases involved in the biosynthesis of arabinans unique to the mycobacterial cell wall. It was further concluded that the uniquely branched terminal Ara₄₅ motif common to both AG and LAM is an essential structural entity for a functional cell wall and, consequently, that the biosynthetic machinery responsible for its synthesis is the effective target of Emb in its role as a potent anti-tuberculosis drug.

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1 The abbreviations used are: Emb, ethambutol; AG, arabino-galactan; Ara₄₅, arabinofuranose; FAB, fast atom bombardment; GC, gas chromatography; HPAEC, high pH anion exchange chromatography; LAM, lipoarabinomannan; mLAM, LAM from M. smegmatis mc²155; LM, lipo-mannnan; mLm, LM from M. smegmatis mc²155; mAGP, mycolylarabinogalactan-peptidoglycan; MALDI, matrix-assisted laser desorption; MIC, minimal inhibitory concentration; MS, mass spectrometry; PAS, periodic acid Schiff; PAGE, polyacrylamide gel electrophoresis.
μg/ml) in media containing increasing concentrations of Emb and investigated the effect of Emb on its arabinan synthesis using a similar approach (Mikusová et al., 1995). It was found that when grown in the presence of 10 μg/ml Emb, the resistant mutant made “normal” cell wall AG but “truncated” LAM of smaller size, indicating a tolerable level of partial inhibition in the selective synthesis of the arabinan of LAM. Thus, despite apparent similarity in their arabinan structures, the biogenesis of AG and LAM may be sufficiently distinct and involve an intricate array of arabinofuranosyltransferases with various degrees of sensitivity to Emb.

In this study, the effect of Emb on the synthesis of arabinan was further inferred from detailed biochemical characterization of both AG and LAM synthesized by the resistant mutant in the presence of varying concentrations of Emb. We demonstrated that the degree of truncation in LAM was dependent on the Emb dose applied and provide a tentative structural basis on which the differential effects of Emb on AG and LAM synthesis may be rationalized. In addition, the identification of similarly truncated LAM in Emb-resistant recombinant strain transfected with defined genetic elements now allows the cloning of specific arabinosyltransferases, which constitute the primary site of action of Emb, thus paving the way for a better understanding of mycobacterial arabinan biosynthesis and rationalization of the role of Emb as an anti-tuberculosis drug.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Transformants—**Plasmid pAEB-148 consists of an 11-kilobase pair partial Sau3AI fragment of Mycobacterium avium DNA cloned into the BamHI site of pMD31.² It contains embR, embA, and embB genes that encode Emb resistance. The plasmid was purified from Escherichia coli strain XL1-Blue (Strategene) and used to electroporate M. smegmatis mc155 (Snapper et al., 1990) by standard procedures (Jacobs et al., 1991). Transformants were selected on 7H11 agar medium containing 10 μg/ml Kan and 1 μg/ml Emb with incubation at 37 °C for 3 days. The resultant recombinant strain was designated AEB-148.

Growth of M. smegmatis mc155 and Emb-resistant Mutant—M. smegmatis mc155 was grown and harvested as described previously for other Mycobacterium sp. (Kho et al., 1995). The Emb-resistant strain KM (MIC = 50 μg/ml) (Mikusová et al., 1995) was propagated on 7H11 plates in the presence of 10 μg/ml Emb as described. Isolated colonies were then grown in 5 ml of 7H11 broth for 24 h at 37 °C on a shaker. Large cultures were initiated by using 100 μl of the 7H11 broth culture to inoculate 5 ml of glycerol-alanine salts medium containing Emb. After 24 h at 37 °C, 1 ml of the glycerol-alanine salts culture was transferred to a liter of glycerol-alanine salts medium containing 10 μg/ml Kan and 1 μg/ml Emb and grown at 37 °C until late log phase. About 5 g of cells were obtained from 2 liters of culture, although the yield varied with the Emb concentrations. At a higher concentration of Emb (25 μg/ml), the culture had to be incubated for 96 h in order to obtain only 2.6 g of cells.

**Extraction of LAM/Lipomannan (LM) and AG—**The cell pellet was first delipidated with absolute ethanol at 70 °C, followed by 2:1 chloroform:methanol at 60 °C, and then extracted with 50% ethanol. The supernatant was concentrated and partitioned between phenol and water (Chatterjee et al., 1992a). The aqueous layer, which contained the majority of cellular LAM and LM was freeze-dried and used for further purification. Cell walls (mAGP) were prepared by sonication of the bacterial pellet in phosphate-buffered saline containing 2% SDS, followed by extensive washing of the insoluble residue in the same buffer. AG was base-solubilized from the mAGP preparation as described (Duff et al., 1990).

**Hydrophobic Interaction Column Chromatography—**The octyl-Sepharose (Pharmacia Biotech Inc.) column (10 × 1 cm) was prepared by equilibrating octyl-Sepharose in 0.1 M sodium acetate, pH 4.7, containing 15% n-propanol. LAM/AG extracts were reconstituted and applied to the column in the equilibrating buffer. After washing with 2 column volumes of this initial buffer to remove nonbound free glycans, LAM/AG was recovered by batch elution with 40 and 65% n-propanol (Leopold and Fischer, 1980). The two fractions were concentrated, pooled, and dialyzed against running water. The same octyl-Sepharose procedures were also used to separate the mannan core from free oligoarabinosides after endoarabinanase digestion of LAM.

**Size Fractonation, SDS-PAGE, and Immunoblotting—**The Sephacryl S-200 (Pharmacia) column was prepared by washing and suspending the gel in a buffer containing 0.2 M NaCl, 0.25% deoxycholate, 1 mM EDTA, 0.02% sodium azide, and 10 mM Tris, pH 8.0 (Chatterjee et al., 1992a). SDS-PAGE was used to analyze the elution profile of fractions containing LAM and LM, which were then pooled accordingly and dialyzed at 37 °C without detergent followed by water for several days. LAM/AG thus recovered was reanalyzed by SDS-PAGE to check for purity prior to detailed analysis. SDS-PAGE, silver-periodic acid Schiff (PAS) staining, and immunoblotting using monoclonal antibody CS-35 were performed essentially as described (Prinz et al., 1990). Sample concentrations were maintained at 1–2 μg in the sample buffer.

**Endoarabinanase Digestion and Subsequent Analyses—**The selective growth of a soil microorganism, Cellulomonas gelida, on an arabinogalactan-containing medium and the isolation and characterization of an extracellular endoarabinanase have been described (McNeil et al., 1994). For complete digestion of LAM, the reaction mixture was typically incubated for 24 h at 37 °C, after which an aliquot was withdrawn and analyzed by SDS-PAGE. The productivity of the enzyme was judged by its electrophoretic mobility compared with LM. If necessary, a fresh aliquot of enzyme was added, and the reaction mixture was left for another 24–48 h. The digestion product mixtures that contained both the mannan core and released oligoarabinosides were analyzed directly by Dionex analytical HPAEC and, after peracetylation, by FAB MS. Alternatively, the oligoarabinosides were completely removed from the mannan core by Bio-Gel Bio-Gel-A-50 sizing column, followed by octyl-Sepharose column chromatography.

Analytical HPAEC was performed on a Dionex CarboPac PA-1 column, and the oligosaccharides were detected with a pulse-amperometric detector (PAD-II) connected to an HP 3390A integrator. Sample was eluted at 1 ml/min with 100 mM NaOH for 2 min, followed by a linear gradient of 0–250 mM sodium acetate, 100 mM NaOH in 40 min. Peracetylation was performed at 80 °C for 2 h using pyridine:acetic anhydride (1:1).

FAB mass spectra were obtained using a VG Analytical Autospec mass spectrometer fitted with a cesium ion gun operated at 25–30 kV. Data acquisition and processing were performed using the Opus® software. Peracetylated samples were dissolved in methanol, and monothiglycerol was used as matrix.

**Monosaccharide Composition and Linkage Analysis—**Alditol acetates were prepared by partial methylation of the fully methylated alditol acetates for GC-MS analysis were prepared from native sample and the permethyl derivatives, respectively, according to the procedures described by Albersheim et al. (1987). LAM/AG was permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell et al. (1994).

GC of the alditol acetates was performed on an HP gas chromatograph model 5890 fitted with a SGE BPF70 fused silica capillary column (0.25 m, 0.25-μm film thickness, 0.22-mm inner diameter) using a temperature gradient of 100 °C for 1 min, 30 °C/min to 200 °C, and then 4 °C/min to 260 °C. GC-MS of the partially methylated alditol acetates was carried out using an HP gas chromatograph model 5890 connected to an HP 5790 mass selective detector. Sample was dissolved in acetone prior to injection on a DB-5 fused silica capillary column (30 m, 0.25-μm film thickness, 0.25-mm inner diameter, J & W Scientific) at 280°C. The temperature was then increased to 180 °C over 20 min and then increased to 280°C at 10 °C/min.

**NMR Spectroscopy—**¹H NMR (DEPT) spectroscopy was performed with a Bruker AM 500 spectrometer. Spectra were obtained (in D₂O) at 500 MHz.

**MALDI MS—**MALDI-time-of-flight MS was performed with an HP LDI 1700XP mass spectrometer operated in the positive ion mode at 30 kV and a pressure of 6 × 10⁻⁷ torr. The mass spectrometer was calibrated with a mixture of glucose oligomers (degree of polymerization between 3 and 20). Aqueous solutions of samples (10 μg/ml) were diluted 1:5 with aqueous 50% acetonitrile containing 100 mM 2,5-dihydroxybenzoic acid and 30 mM 1-hydroxyisouquinoline (Mohr et al., 1995), and a portion (0.5 μl) was applied to the probe tip of the mass spectrometer. Samples were desorbed from the probe tip with a nitrogen laser (λ 337 nm) having a pulse width of 3 ns and delivering approximately 16 μJ of energy/laser pulse.

² Belanger, A. E., Besra, G. S., Ford, M. E., Mikusová, K., Belisle, J. T., Brennan, P. J., and Inamine, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A., in press.
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Results

Size Heterogeneity in the Truncated LAM—The existence of a truncated variant of LAM was first recognized when the KM, Emb-resistant mutant (MIC = 50 μg/ml) was grown at 10 μg/ml Emb and metabolically radiolabeled with [U-14C]glucose after 8 h of growth (Mikušová et al., 1995). A 14C-labeled product in the partially purified LAM-LM fraction was shown to migrate on SDS-PAGE and a Bio-Gel P-100 sizing column at a mobility corresponding to a size intermediate between that of normal LAM synthesized by the non-Emb-treated parent strain and LM, which is devoid of arabinan. This novel product was identified as a truncated variant of LAM based on its monosaccharide composition and positive recognition by the LAM-specific monoclonal antibody CS-35 (Mikušová et al., 1995).

To confirm and further delineate the structural identity of this truncated variant, KM was grown at the same Emb concentration (10 μg/ml, hereafter referred to as KM10) and after 24 h, LAM was extracted and purified according to established protocols (Hunter and Brennan, 1990; Chatterjee et al., 1992a). After the final stage of purification on an octyl-Sepharose column to remove free glycan contaminants, fractions containing LAM and LM were pooled and size-fractionated on a Sephacryl S-200 column. Normal LAM, as synthesized by Emb-susceptible *mc*155 and other *Mycobacterium* sp. in the absence of Emb, typically eluted in 10–20 successive fractions prior to the elution of LM, and each of these fractions would stain with PAS as a broad diffuse band spanning the same size range on SDS-PAGE (Chatterjee et al., 1992a). However, the corresponding LAM-containing S-200 fractions from KM10 gave increasingly “smaller” LAMs on SDS-PAGE, with the last few fractions virtually indistinguishable from LM. Arbitrarily pooled into seven fractions (every four fractions from the column eluates), these truncated LAMs (designated as KM1–KM7) when reanalyzed by SDS-PAGE gave the PAS staining pattern as shown in Fig. 1. Monosaccharide composition analysis on each of these pooled fractions gave an Ara:Man ratio ranging from approximately 3 for KM1 to about 0.3 for KM7 (Table I). This result indicated that KM10 synthesized an extremely heterogeneous population of LAMs with electrophoretic mobilities and corresponding Ara:Man ratios varying from those resembling a “full-size” normal LAM to those similar to LM.

Varying Degrees of Arabinan Truncation—The varying Ara:

![Fig. 1. SDS-PAGE analysis of truncated LAMs synthesized by KM at 10 μg/ml Emb. LAM was visualized by silver-PAS staining.](image)

**TABLE I**

| LAM/LM   | Ara:Man ratio | Mol % |
|----------|---------------|-------|
| mcLAM    | 3.0           |       |
| Near normal | 4.9        |       |
| KM1      | 2.7           | 1.2   |
| KM2      | 2.5           | 3.7   |
| Midsize  | 43.7          |       |
| KM3      | 2.0           | 8.7   |
| KM4      | 1.5           | 12.9  |
| KM5      | 0.9           | 22.1  |
| KM6      | 0.4           | 24.2  |
| KM7      | 0.3           | 27.2  |

The molar percentage was defined with respect to the total pool of LAM isolated from KM10 (i.e. KM1–KM7), based on the Man content of each fraction relative to added internal standard on the assumption that each truncated LAM was based on mannan core of identical size. KM1 and KM2, KM3–KM5, and KM6 and KM7 were further categorized as near normal, midsize, and extremely truncated, respectively. The total molar percentage of each category was summed from the values of the relevant fractions contained.

Man ratios in KM1–KM7 suggested that the size heterogeneity exhibited by the truncated LAMs was a direct consequence of varying degrees of truncation in the arabinan, assuming that all the truncated LAMs synthesized by KM contained the same mannan core of normal length. We have previously shown that the mannan core of LAM can be obtained intact after exhaustive digestion of deacylated LAM with a crude endoarabinanase extract from *C. gelida* (Chatterjee et al., 1993). To provide a direct comparison and visualization on SDS-PAGE, KM3, KM5, and LM from the parent *mc*155 strain (mcLAM) were each digested with endoarabinanase without prior deacylation. The truncated KM3 and KM5 gave similar diffuse bands running at the same position as LM standard from *mc*155 (mcLAM) after a 24-h digestion (Fig. 2A, lanes 6 and 7), whereas mcLAM typically required several repeated digestions (with fresh aliquots of enzyme) but eventually also gave a same-size band on the gel. The mannan cores from KM3 and mcLAM were purified from released free oligosaccharinides by octyl-Sepharose column chromatography, and subsequent monosaccharide composition analysis confirmed that each contained an Ara:Man ratio of about 0.05.

After deacylation and permethylation, KM3 mannan core, mcLAM mannan core, and mcLAM all gave similar MALDI spectra with a broad cluster of peaks centered around m/z 6000 (Fig. 3). Within the cluster, individual peaks were each separated by about 204 mass units, indicating a mannan core/LM heterogeneous in the number of Man residues. It can be inferred from the spectra that the most abundant species fell within m/z 5000–6500, corresponding to approximately 22–29 residues of Man with one single Ara residue. This would translate into a molecular mass of ~5 kDa for a native mannan core/LM, containing 28 residues of Man and acylated with palmitic and tuberculostearic acids.

The MALDI spectra and SDS-PAGE analysis on the mannan
cores indicated that all the truncated LAMs were indeed based on a mannan core of similar size. Taking an average of 26 Man residues for the mannan core, it can be concluded from the Ara:Man ratio (Table I) that KM10 synthesized LAMs with truncated arabinan averaging from about 67 to 8 Ara residues.

The MALDI spectra of the deacylated, permethylated KM1 and KM2 pool gave a broad unresolved peak centered around \( m/z \) 13,000 (Fig. 4A), whereas that of KM3 and KM4 pool centered around \( m/z \) 9,500 (Fig. 4B). This corresponded to a mean size of approximately 45 and 23 Ara residues for the “near normal” and “midsize” truncated LAMs, respectively, with substantial heterogeneity and significant variation in the mean distribution depending on the purification procedures and pooling of sample fractions. It may be further estimated that the molecular mass of a normal, diacylated mcLAM in its native form is ~15 kDa, with about 78 Ara and 26 Man residues. Although an accurate quantification was not possible, the Man content as shown by monosaccharide composition analysis (Table I) clearly indicated that the near normal KM1 and KM2 only constitute about 5% of the total LAM made by KM10, whereas the midsize KM3 to KM5 and the extremely truncated KM6 and KM7 each contributed about 43 and 51%, respectively. Thus, at a concentration of 10 \( \mu \)g/ml Emb, Emb affected a drastic and overall truncation in the arabinan portion of LAM synthesized by KM, with no apparent effect on the mannan core.

**Emb Dose-dependent Truncation of Arabinan in LAM and AG—** In agreement with previous results (Mikušová et al., 1995), the AG synthesized by KM10 showed no apparent difference from the normal AG of *M. smegmatis* mc²155 (Daffe et al., 1993) as indicated by the \(^{13}C\)-NMR “fingerprint” (Fig. 5B). Direct monosaccharide composition analysis on the base-solubilized AG as well as the mAGP complex from KM10 gave an Ara:Gal ratio of 2.7, comparable with that of mc²155. However, similar analysis on mAGP from KM grown at 15 and 25 \( \mu \)g/ml Emb (referred to as KM₁₅ and KM₂₅, respectively) showed a significant decrease in this value, 2.3 for KM₁₅ and 1.65 for KM₂₅. At 25 \( \mu \)g/ml Emb, the growth rate of KM was also severely affected, giving less yield (wt weight of harvested cells) from a longer period of growth, as well as loss of acid fastness. Thus, although KM showed no inhibition in the synthesis of cell wall AG at 10 \( \mu \)g/ml Emb, at higher Emb concen-
Truncated LAMs of M. smegmatis

**Fig. 5.** $^{13}$C NMR DEPT spectra of the base-solubilized cell wall AG of AEB-148 (A) and KM$_{10}$ (B), showing a fingerprint indistinguishable from each other and from that of M. smegmatis (Daffe et al., 1990). The major C-1 signals appeared between $\delta$ 109 and $\delta$ 108, corresponding to $\alpha$-Araf and galactofuranose residues. The signals at $\delta$ 106.8 and $\delta$ 106.6 were attributable to the C-1 of 1-linked Araf residues, confirming the presence of terminal Araf motif, the only site where a 2-linked Araf can be present. The resonances at $\delta$ 101.9 and $\delta$ 101.8 were consistent with the presence of $\beta$-Araf. In addition, the signals at $\delta$ 88.2 and $\delta$ 87.9 were the C-2 resonances of the 2-linked-$\alpha$-Araf.

The extremely truncated LAMs contained only short 5-arabinan chains extending from the mannan core. Thus, the extremely truncated LAMs from KM were found to be similar to that of mcLAM (Khoo et al., 1995), indicating the presence of a full complement of previously identified arabinan motifs (Chatterjee et al., 1991; Khoo et al., 1995). Typically, the branched Araf and linear Araf motifs constitute the two main types of nonreducing termini in mcLAM, i.e. t-Araβ1-2Araα1-5(t(t-Araβ1-2Araα1-3)Araα1-5)Araα1-5Araα1-5 motif.

In the extremely truncated LAMs such as KM6 and KM7 from KM$_{10}$, as well as the unresolved LAM/LM fraction from KM$_{25}$, only t-Ara and 5-Ara were present at significant levels, suggesting that a certain minimal length of t-Ara units in the arabinan chain was probably needed before any appreciable branching and subsequent $\beta$-Ara termination could occur. Thus, the extremely truncated LAMs contained only short stubs of linear t-Ara to 3,5-linked Ara (about 4:1) would reflect the extent of branching on the rest of the terminal 5-Ara chain. 2-linked Ara was, however, consistently found to be slightly less than t-Ara, indicating that, typically, about 10–20% of the arabinan termini in mcLAM did not terminate with the Araβ1-2Araα1-5 motif.

The significant amount of 3,5-linked Ara present in LAMs as truncated as those from KM$_{15}$ and KM5 suggested that branching was not restricted to the arabinan distal from its attachment site on the mannan core, although it was not possible to ascertain the exact location of the first branched point. In fact, the slightly lower ratio of 5-linked Ara to 3,5-linked Ara (about 3:1 versus 4:1 in normal mcLAM) suggested that these subpopulations of truncated LAMs were more branched as compared with a full-size normal mcLAM. In addition, the apparent reduction in the molar ratio of 2-linked Ara relative to terminal Ara was indicative of a higher proportion (>20%) of the termini not being terminated with a $\beta$-Ara at position 2. Emb-resistant Arabinosyltransferase in AEB-148—The synthesis of a full spectrum of truncated LAMs in KM$_{10}$ indicated that KM has a functional machinery for the synthesis of arabinan and that each of the arabinosyltransferases required was completely or partially resistant to Emb as a consequence of mutation in one or more genes encoding for these enzymes. Indeed, a 9.5-kilobase pair DNA fragment from M. avium that encodes for the emb Emr resistance region has been completely sequenced and shown to be associated with an Emb-resistant

The synthesis of a full spectrum of truncated LAMs in KM$_{10}$ indicated that KM has a functional machinery for the synthesis of arabinan and that each of the arabinosyltransferases required was completely or partially resistant to Emb as a consequence of mutation in one or more genes encoding for these enzymes. Indeed, a 9.5-kilobase pair DNA fragment from M. avium that encodes for the emb Emr resistance region has been completely sequenced and shown to be associated with an Emb-resistant

Finally, when KM was grown at 1 $\mu$g/ml Emb, it yielded LAM similar in size to the normal or near normal LAM, with an Ara:Man ratio of 2.7.

Accordingly, KM, with a MIC of 50 $\mu$g/ml Emb, synthesized an extremely heterogeneous population of LAM when grown on 10 $\mu$g/ml Emb, ranging in size from near normal to those severely truncated in its arabinan component. The mean distribution of this size heterogeneity could be shifted to either extreme by varying the concentration of Emb, such that at high Emb concentrations, severe inhibition of arabinan synthesis resulted in the near absence of LAM as well as apparent truncation of the cell wall AG.

**Arabinan Motifs in Truncated LAM—**A comparison of the linkage analysis profiles of the various truncated LAMs from KM (data not shown) showed that the relative amount of terminal Man, 6-linked Man, and 2,6-linked Man in each sample was similar, consistent with the earlier conclusion that all truncated LAMs contained the same $\alpha$1–6 mannan core, with approximately half of the 6-linked Man in the chain being further substituted with single Man residues at position 2 (Khoo et al., 1995). The molar ratio of terminal Araf, 2-linked Araf, 5-linked Araf, and 3,5-linked Araf in the near normal and midsize truncated LAMs from KM were found to be similar to that of mcLAM (Khoo et al., 1995), indicating the presence of a full complement of previously identified arabinan motifs (Chatterjee et al., 1991; Khoo et al., 1995). In the extremely truncated LAMs, the Ara:Man ratio of KM6 and KM7 (Table I), it was estimated that such linear arabinan chains may consist of up to about 10 Ara residues.
arabinosyltransferase activity in a cell-free assay for arabinan biosynthesis.\textsuperscript{2} When Emb-susceptible mc\textsuperscript{155} was electroporated with plasmid pAEB-148 containing this genetic element, the resultant recombinant strain (designated AEB-148) was shown to be resistant to Emb with a MIC of 2.5 $\mu$g/ml. Due to a lower MIC as compared with KM, AEB-148 could only be grown at 1 $\mu$g/ml Emb, and the LAM and AG synthesized were similarly purified and analyzed.

The base-solubilized AG from AEB-148 gave a $^{13}$C NMR fingerprint (Fig. 5A) identical to those of \textit{M. smegmatis} and KM\textsubscript{10}, as well as a normal Ara:Gal ratio, confirming the synthesis of normal AG. As shown in Fig. 2, AEB-148 clearly synthesized midsize truncated LAM comparable with KM3–KM5 and intermediate between those of KM\textsubscript{1} and KM\textsubscript{15}. Monosaccharide composition analysis of arbitrarily pooled successive fractions from S-200 sizing column indicated an Ara:Man ratio of 1:5 equivalents.\textsuperscript{3} Accordingly, AG digested with LM and other mannan cores (data not shown).

**Structural Basis for Arabinan Truncation**—In addition to the mannan core, we have previously shown that mcLAM typically yielded Ara\textsubscript{2}, Ara\textsubscript{4}, and Ara\textsubscript{6} as the major digestion products when treated with the crude endoarabinanase mixture (Kho et al., 1995), whereas similar digestion of AG gave primarily Ara\textsubscript{2} and Ara\textsubscript{6} with very little Ara\textsubscript{4} (McNeil et al., 1994), consistent with the lack of linear Ara\textsubscript{4} termini in the cell wall AG (Beara et al., 1995). Using AG as substrate, the crude endoarabinanase preparation has since been fractionated to yield a preparation enriched with enzyme activity specific for endoarabinanase.\textsuperscript{3} Accordingly, AG digested with this partially purified endoarabinanase gave only Ara\textsubscript{6} as the dominant product, whereas mcLAM gave a variety of products dominated by Ara\textsubscript{4} and Ara\textsubscript{6} peaks, as visualized directly by the Dionex HPAEC mapping of the digestion mixtures (Fig. 6).

As expected, the near normal truncated LAMs yielded a HPAEC profile similar to that of normal mcLAM (Fig. 6A), whereas no significant peak was observed when the digestion mixtures of those extremely truncated ones such as those from KM\textsubscript{25} were analyzed. Interestingly, the midsize truncated LAMs from KM\textsubscript{10} gave a distinctive profile (Fig. 6B) with the Ara\textsubscript{4} peak only half as abundant relative to Ara\textsubscript{6}, while the digested truncated LAMs from AEB-148 gave only the Ara\textsubscript{6} peak with very little Ara\textsubscript{4} (Fig. 6C). FAB MS analysis of the peracetyl derivatives of the total digestion products showed that those of KM\textsubscript{10} (Fig. 7A) were dominated by molecular ion signals at m/z 989 and 1421, corresponding to [M + Na]\textsuperscript{+} of Ara\textsubscript{4} and Ara\textsubscript{6}, respectively, as expected. Although both peaks were also present in the corresponding spectrum of the truncated LAM from AEB-148 (Fig. 7B), it was clear that there was a significant reduction in the relative amount of Ara\textsubscript{4}.

It was thus concluded that although both linear Ara\textsubscript{4} and branched Ara\textsubscript{6} termini were synthesized by the resistant mutant KM and readily detectable by HPAEC and FAB MS analysis, there is a significant reduction in the relative amount of the linear termini in the midsize truncated LAMs. This apparent lack of linear Ara\textsubscript{4} terminal motifs was particularly severe in the truncated LAM from the Emb-resistant recombinant AEB-148. The truncation in arabinan in the LAMs of KM and AEB-148 may therefore be best rationalized as a partial inhibition in the synthesis of the properly terminated linear a1–5 arabinan chains (Fig. 8).

3 J. Xin and M. McNeil, manuscript in preparation.
Truncated LAMs of M. smegmatis

Ara₄ termini, may reflect the stringent requirement for subsequent mycolylation of the AG in making a functional mAGP cell wall complex. In contrast, a high proportion of the arabinan chains in LAM terminate as linear Ara₄ instead of the branched Ara₆ (Chatterjee et al., 1991, 1993; Kho et al., 1995). Thus, the major digestion product of AG using the partially purified endoarabinanase is the Ara₆, whereas digestion of LAM yields primarily Ara₄ and Ara₆ in approximately equal amounts. By direct Dionex HPAEC analysis of the digestion products coupled with definitive FAB MS analysis, we have now shown that the near normal LAM and the AG synthesized by the Emb-resistant KM strain indeed comprise the Ara₄/Ara₆ and Ara₆ motifs, respectively, in the expected ratios. More significantly, the truncated LAMs from KM was found to have a reduced Ara₄:Ara₆ ratio, whereas the truncated LAM from AEB-148 gave very little Ara₄ relative to Ara₆. Thus, a major conclusion arising from this work is that, in addition to AG, Emb inhibits the synthesis of the arabinans in LAM in a dose-dependent manner and that truncation or reduction in size is primarily a consequence of incomplete elaboration of the terminal linear Ara₄ motif, which is not present in AG (Fig. 8).

The recombinant AEB-148 strain (MIC of 2.5 μg/ml Emb) was derived from electroporating Emb-susceptible M. smegmatis mc²155 (MIC of 0.25 μg/ml Emb) with a plasmid pAEB-148 containing the emb region from a clinical isolate of M. avium. Sequence analysis indicated that there are three genes in this region, embR, embA, and embB, and that the translationally coupled embA and embB genes are necessary and sufficient to confer an Emb-resistant phenotype when expressed in M. smegmatis on a multicopy vector. A cell-free system developed by Lee et al. (1995) was previously shown to be effective in using the lipid carrier, decaprenylphosphoarabinosine, as a donor of arabinose in the polymerization of arabinan. The incorporation of radiolabeled Ara into a polymer of arabinan was inhibited to a maximum level of 70% when increasing amounts of Emb were added to the reaction mixture containing membrane fractions of M. smegmatis mc²155. A comparative study showed that similar extracts from AEB-148 and KM were only inhibited to about 35 and 60%, respectively. Although it is difficult to extrapolate the extent of inhibition to the in vivo biosynthesis system, these results indicated that the embB and embA genes in pAEB-148 are associated with high level Emb-resistant arabinosyltransferase activity. Transfection with these genes allowed AEB-148 to synthesize normal AG and slightly truncated LAM with a significant decrease in the relative level of the Ara₄ motif.

The differential mode of action of Emb on AG and LAM may thus be best rationalized by implicating the arabinosyltransferases associated with the machinery making the Ara₆ motif as the effective target of Emb. These are likely to be, or include, the embA and embB gene products such that when they are overexpressed, the cell can make functional AG and survive at a higher MIC level of Emb. By virtue of the selection strategy, the resistant mutant KM would necessarily make normal and functional AG in order to grow at elevated levels of Emb and is likely to have Emb-resistant homologues of the Ara₆-making arabinosyltransferases arising through mutation. The observation that KM and AEB-148 now make truncated variants of LAM indicated that Emb still exerts partial inhibitory effects on other arabinosyltransferases associated with the general initiation, elongation, and maintenance of a certain normal proportion of Ara₄ relative to Ara₆ in LAM. Thus, at about up to 15 μg/ml Emb, the prime effect of Emb on KM is to exert an increasing truncation in the linear Ara₄ motif (Fig. 8), but at higher concentrations the general truncation in arabinan becomes more apparent, effecting a reduction in size in both LAM and AG. A lower MIC of Emb for AEB-148 as compared with KM is consistent with the hypothesis that Emb resistance in the former is a consequence of overexpressing the effective target, the neutralization of which is only effective up to 1 μg/ml Emb.

Based on the known structure of arabinan, it may be further speculated that the polymerization of arabinan is essentially an α₁-5 elongation of the arabinan chains punctuated by α₃-branching. The linear terminal Ara₄ motif is a consequence of nonbranched termination with β₂-Ara, whereas the terminal Ara₆ motif is the branched counterpart. Thus, Emb may be inhibiting all or most of the arabinosyltransferase involved in the biosynthesis of arabinan, a phenomenon not unexpected given that all individual arabinosyltransferases are likely to recognize and utilize the same donor such as decaprenylphosphoarabinosine and hence contain structurally homologous active sites. The differential effect of Emb in eliciting synthesis of truncated LAMs but normal AG in the resistant strains is a consequence of the differential requirement of these two arabinan-containing components in growth. Selection for growth in culture in the presence of Emb entails that the mutant or recombinant must now be able to make functional AG, whereas a defective LAM is tolerable, at least for in vitro growth. This would translate into a more stringent requirement for the α₃-branching arabinosyltransferase (or the composite biosynthetic machinery specifically required for making Ara₆), a target that needs to neutralize the effects of Emb by overexpression or mutation in order for the cell to grow. In the presence of Emb, the competition between branching and elongation would be distorted in favor of the branching Ara₆ terminal motif, resulting in the phenomenon of truncated LAM in KM and AEB-148.

The observation that a full-size mature LAM is not a requisite for mycobacterial growth in culture does not discount LAM's biological significance in contributing to the survival of
the bacterium in vivo. We argue that in order for LAM to effectively induce and/or suppress proper immune response in the host, a fully functional LAM is required and that most of its function will be critically dependent on the integrity of its terminal arabinan motifs, its exposure on the surface, and perhaps active secretion. A truncated LAM will therefore be likely to be defective in its many known and implicated immunoregulatory roles (Kaplan et al., 1987; Sibley et al., 1988; Chan et al., 1991), such as being the dominant B-cell immunogen (Khanolkar et al., 1989), and possibly in mediating the binding to macrophage mannose receptor (Schlesinger et al., 1994) through incomplete elaboration of the terminal motifs for mannose capping. Although we have not yet extended our studies to M. tuberculosis, our data showed that in the more truncated LAMs there is an overall and selective reduction in the terminal motifs and that these were no longer recognized by the LAM-specific monoclonal antibodies.

Despite the recognition of various Ara motifs, a definitive picture of the intact arabinan in LAM remains elusive. Both monosaccharide composition and linkage analysis have consistently indicated an Ara:Man ratio of about 3. We have now, for the first time, shown that both the mannan core and LM of M. smegmatis consist of about 26 Man residues with considerable heterogeneity. Hence, the arabinan of normal LAM may be inferred to contain more than 70 Ara residues. At such a high mass of ~14 kDa for the intact LAM, mass spectrometry analysis proved to be difficult, and only an unresolved "hump" was observed in the MALDI mass spectra. Although analysis of the native molecule is possible (Venisse et al., 1993), the more hydrophobic permethyl derivatives proved to desorb better. Under the optimized matrix conditions (Mohr et al., 1995), we have been able to obtain MALDI mass spectra for most of the heterogeneous population of truncated LAM, mannan core, and LM at about the 100-µg level or less, with about 1 µg of sample being actually loaded onto the probe tip for each data acquisition. The MALDI MS data of the deacylated, permethylated mannan core and LM not only defined the size but also indicated that heterogeneity in these molecules is associated with the total number of Man residues that were resolved as individual peaks.

Thus, the physicochemical and hence the biological properties of truncated LAM are an average attribute reflecting a heterogeneous population of molecules differing in the total number of Ara and Man residues. By increasing the concentration of Emb, we effectively reduced not only the overall size but also the heterogeneity by restricting the elongation of the arabinan chain. The presence of only a few Ara residues on these molecules has greatly facilitated the structural analysis aimed at defining the attachment site of the arabinan and its immediate chemical settings, the result of which will be reported elsewhere. Finally, the truncated LAMs also function as surrogate biosynthetic intermediates, based on which further genetic manipulation and biochemical characterization may be designed to delineate the intricate array of arabinosyltransferases involved in the biosynthetic pathway.

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