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Characterization of a Specific Arabinosyltransferase Activity Involved in Mycobacterial Arabinan Biosynthesis

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Summary

Mycobacterium smegmatis **strains that contain inactivated EmbA or EmbB proteins are unable to synthesize terminal Araβ1→2Araα1→5(Araβ1→2Araα1→3)Araα1→5Araα1→(Ara_6) motif in the cell wall polysaccharide arabinogalactan. Instead, the mutants contain a linear Araβ1→2Araα1→5Araα1→5Araα1→(Ara_4) motif, suggesting that these proteins are involved in the synthesis or transfer of the disaccharide Araβ1→2Araα1→ to an internal 5-linked Ara. Therefore, we synthesized a linear Araβ1→2Araα1→5Araα1→5Araα1→5Araα1→ with an octyl aglycon as an arabinosyl acceptor in cell-free assays. A facile assay was developed using the chemically synthesized glycan, membrane, and particulate cell wall as the enzyme source, and 5-phosphoribose diphosphate pR[^{14}C]pp as the arabinose donor. The result*s* unequivocally show that two arabinofuranosyl residues were added at the tertiary →5Araα1→ of the synthetic glycan. This activity was undetectable in strains of *M. smegmatis* where *embB* or *embA* had been genetically disrupted. Normal activity could be restored only in the presence of both EmbA and EmbB proteins.**

Introduction

D-arabinofurans are key components in the cell wall of all mycobacterial species [1–3] and are present in two distinct settings. In mycolyl-arabinogalactan (AG), the arabinan is the point of attachment of mycolic acids, long-chain branched fatty acids that are responsible for the low permeability of the bacterial cell wall toward antibiotics [4, 5]. In the cell wall-associated lipoarabinomannan (LAM), the arabinan is nonmycolylated; instead, it is capped with short mannose oligosaccharides in slow-growing *Mycobacterium* or inositol phosphate in *Mycobacterium smegmatis* [6]. The mycobacterial arabinofurans are structurally unique among the polysaccharides known in nature. They are homopolymers with intricate branching pattern and are composed of the biologically rare sugar D-Ara*f*. Although D-Ara*f* is involved in the biosynthesis of ketodeoxyoctulosonic acid (KDO) [7] as a component of lipooligosaccharides, it is rarely found outside the *Actinomycetales*. The current structural model of AG-arabinan is depicted as a composite of three Ara_{22}mers directly attached to the galactan backbone evolving from a seminal structural study [8].

The nonreducing termini of the AG-arabinan has a characteristic structural feature which is a terminal hexaarabinofuranosyl (Ara_6) motif, Ara*f*β1→2Ara*f*α1→5(Ara*f*β1→2Ara*f*α1→3)Ara*f*α1→5Ara*f*α1→, where both the terminal β-Ara*f* and the penultimate 2-α-Ara*f* serve as the anchoring points for the mycolic acids. In contrast, the nonreducing termini of the arabinan in LAM, a linear tetraarabinofuranosyl (Ara_4) motif, Ara*f*β1→2Ara*f*α1→5Ara*f*α1→5Ara*f*α1→, is known to coexist with the branched Ara_6 termini. Through varying degree of mannose capping at its nonreducing terminal β-Ara*f*, the arabinan in LAM plays pivotal immunomodulatory functions in host-pathogen interactions and disease outcome.

Mycobacterial D-Ara*f* originate from the pentose phosphate pathway/hexose monophosphate shunt and the donor of the D-arabinofuranosyl residues is decaprenyl phosphoryl-D-arabinose (C_{50}-P-Ara*f*; DPA) [9], which is formed from 5-phospho-D-ribose diphosphate [10] and not a nucleotide sugar. Given the complexity of the arabinan in AG, a number of arabinosyltransferases that utilize DPA are likely to exist.

Ethambutol (EMB), a drug extensively used in combination therapy against multidrug-resistant tuberculosis, has been shown to affect arabinan metabolism [11–13], although its precise mode of action is unknown. During the course of identifying the target and mechanisms of resistance of EMB, Belanger et al. [14] showed that the proteins encoded by the mycobacterial genes *embA* and *embB* conferred EMB resistance in *M. smegmatis*. Importantly, it was also shown that EMB inhibited in vitro formation of arabinan to about 50% in an assay using endogenous acceptors and chemically synthesized DPA as the donor of Ara*f* units. This inhibition was lessened in strains overexpressing *embB* [14], suggesting that the proteins encoded by the *emb* genes catalyzed the addition of Ara*f* residues to nascent arabinan.

Disrupted mutants of either *embA* or *embB* of *M. smegmatis* proved to be viable. However, arabinosylation of AG in each mutant was considerably reduced and, specifically, the formation of the nonreducing terminal disaccharide β-D-Ara*f*-(1→2)-α-D-Ara*f* characteristic of the arabinan of AG was markedly diminished [15], again suggesting that the Emb proteins were intimately associated with arabinan synthesis and could be arabinosyltransferases.

Although the structural data from previous experiments were sound, we lacked the ability to directly assay arabinosyltransferase activity. Therefore, we designed a molecule to act as an acceptor of enzymatically transferred Ara*f* residues that would mimic the native structure of mycobacterial AG, and the terminal Ara_6 in particular. This molecule is a linear pentamer with the structure **27**, Araβ1→2Araα1→5Araα1→5Araα1→5Araα1, as its octyl glycoside. The pentamer **27** was initially designed with two objectives in mind. (1) There is only one site

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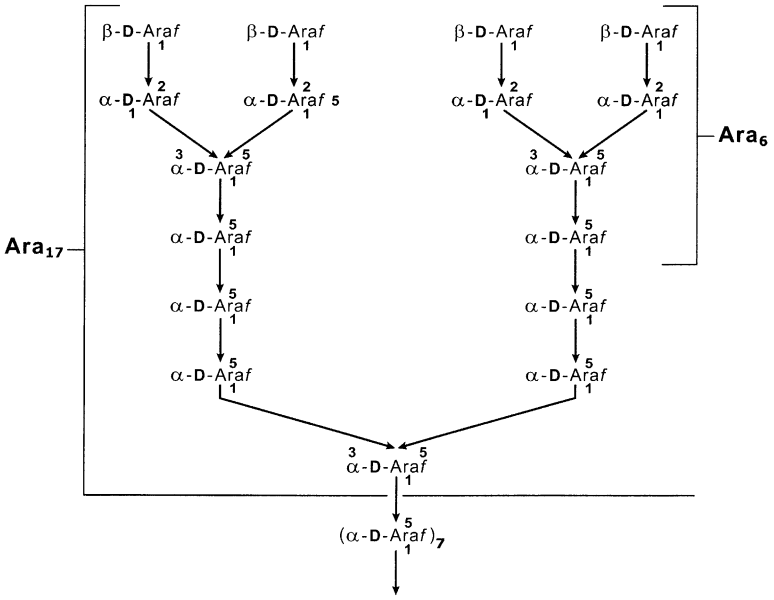

Figure 1. Structure of Arabinogalactan

Although the termini in AG comprising Ara_6 and Ara_{17} have been well characterized, the inner extension of Araf-(1→5)-α-Araf is unknown.

available for the physiologically relevant arabinosyltransferases to act. (2) The enzymatically synthesized product can be digested with endoarabinanase isolated from *Cellulomonas gelida* to yield the well-characterized Ara_6 motif (Figure 1), allowing unambiguous identification of the product's structure [16]. Use of this pentamer would also allow us to probe the question of whether EmbA and EmbB were directly involved in the terminal Ara_6 formation and whether these enzymes were functionally coupled [17].

In this study, we demonstrate unambiguously that a mycobacterial cell-free preparation from wild-type *M. smegmatis* was able to catalyze β-D-Araf-(1→2)-α-D-Araf transferase activity to a synthetic substrate. This transferase activity was not detectable in strains of *M. smegmatis* where either the *embA* or *embB* gene has been genetically disrupted and was restored by mixing the enzyme source from the two disrupted strains.

Results

A synthetic strategy of sequential addition of Araf residues to build an arabinosyl acceptor (compound **27**) was employed, using well-established thioglycoside glycosylation reactions [18]. Protecting groups were chosen such that once the oligosaccharide is assembled, a one-step debenzylation would afford the desired pentamer **27**. Most of the manipulations of protection and deprotection were carried out at the monosaccharide level of the synthetic strategy, alleviating difficulties in handling reaction/purification after assembly of the pentasaccharide.

Synthesis of Building Block 8

Commercially available hemiacetal 2,3,5-tri-*O*-benzyl-D-arabinofuranose was acetylated and then converted to its *p*-thiocresol derivative in the presence of boron trifluoride ether to give compound **3**. The *p*-cresyl 2,3,5-tri-*O*-benzyl-1-thio-α-D-arabinofuranoside **3** underwent thioglycoside glycosylation with *n*-octanol in the presence of *N*-iodosuccinimide and silver trifluoromethanesulfonate to yield the octyl glycoside (**4-1** and **4-2**), predominantly as an α:β anomeric mixture (29.6% β, 32.3% α after column chromatography). The benzyl protecting groups were removed by hydrogenolysis; the 5-OH was selectively tritylated to form compound **6**, which was rebenzylated and then the trityl group removed using 5% trifluoroacetic acid (TFA) to yield the building block/acceptor **8** in 41.6% yield after chromatography (Figure 2).

Synthesis of Building Block 13

The C-5 OH of D-arabinose could be easily protected by treatment with *t*-butyldiphenylsilyl chloride (TBDPSCl) to give compound **9** in moderate yield. Acetylation and *p*-thiocresylation gave compound **11**. Deacetylation and benzylation afforded the fully protected building block donor **13**, which could be selectively deprotected at the 5-position for further modification. Except for the initial step of selective protection of D-arabinose at the 5-OH group with TBDPS, compounds **10**, **11**, **12**, and **13** were all obtained in very high yields, thus supporting the judicious choice of synthetic routes (Figure 2).

Synthesis of Building Block 23

The 1-OH and 2-OH of compound **9** were protected as an isopropylidene acetal using acetone and anhydrous cupric sulfate. Compound **18** was obtained in over 90% yield. The TBDPS group was removed to give **19**, which was benzylated at the 3- and 5-positions to give fully protected **20**. The isopropylidene group was removed with acetic acid and the resulting compound **21** was converted to the building block **23** in two steps (Figure 2).

Synthesis of Compound 27

With the three donors in hand, preparation of the target oligosaccharide was accomplished as described in Figure 3. Tetrasaccharide **24** was assembled using compounds **13** and **23** as the donors. Removal of the benzoyl group converted compound **24** to a suitable acceptor for *O*-2 glycosylation. The fully protected pentamer with the

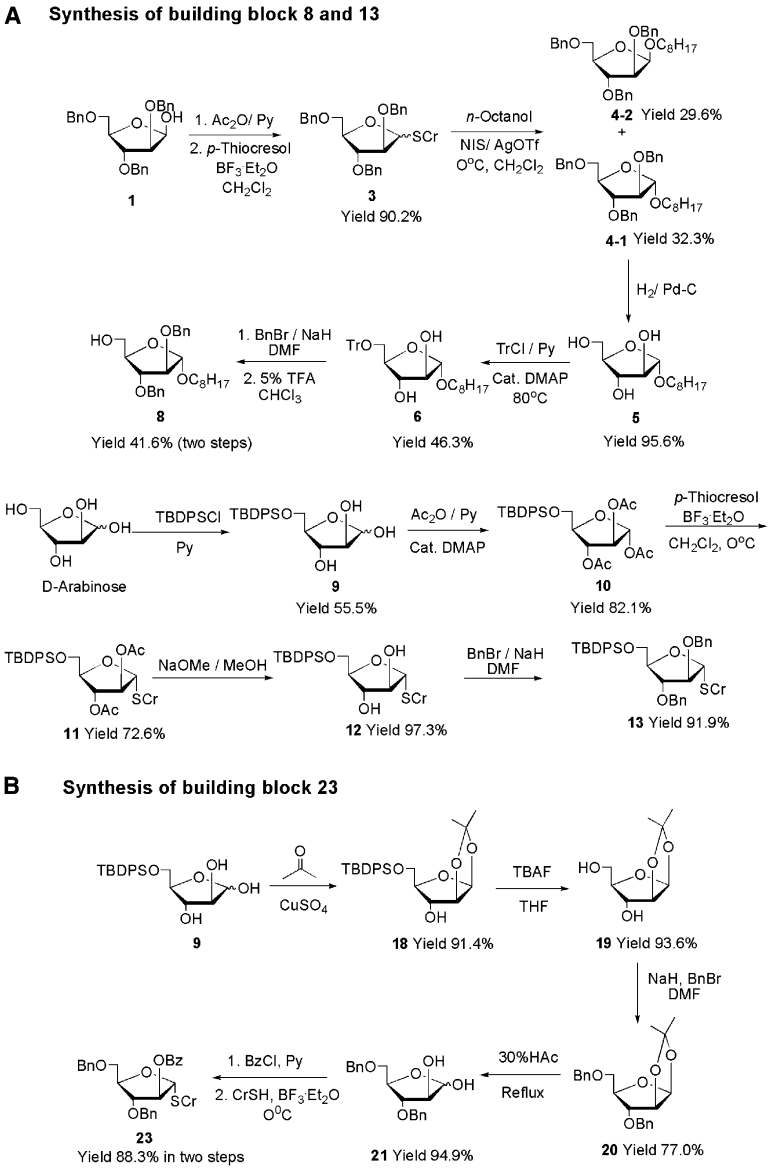

Figure 2. Synthesis of Key Intermediates

Chemical synthesis of the building blocks used in the synthesis of the target pentamer. The reagents used were added at the indicated intermediates. Key intermediates were compounds **8** and **13** (A) and **23** (B). The synthetic procedures are described in Experimental Procedures. Most of the reactions are based on the use of suitable protecting groups described in the literature (cited in the text).

terminal sugar in β-anomeric configuration was obtained when the final glycosylation was performed at −78°C in the presence of *N*-iodosuccinimide and silver trifluoromethanesulfonate. The structures of all compounds were confirmed by NMR (^{1}H and ^{13}C) and, specifically, introduction of the β-anomeric Ara*f* residue in compound **26** was confirmed by chemical shift $\delta_{H\text{-}1}$ 5.10; $\delta_{C\text{-}1}$ 100.6, in contrast to α-anomerics centered at $\delta_{C\text{-}1}$ 106.7 (3), 105.3, respectively. Electrospray mass spectral analysis of **27** yielded a positive ion at m/z 813 $(M + Na)^+$ corroborating for $C_{33}H_{58}O_{21}$. Linkage analysis (gas chromatography-mass spectrometry) indicated that compound **27** had t-Ara*f*, 2-Ara*f*, and 5-Ara*f*.

Enzymatic Assays

The pentamer **27** was tested for its ability to act as an acceptor substrate for arabinosyltransferase activity in a cell-free assay using p[^{14}C]Rpp (which is converted by the cell wall membrane fraction to DPA) as an indirect arabinosyl donor. When a mixture of the membrane- and cell wall-enriched fraction was incubated with p[^{14}C]Rpp and compound **27**, a reasonable amount (4%–6% of the p[^{14}C]Rpp used) of radiolabeled material was found in the 1-butanol extract with a significant amount of radioactivity remaining in the water layer. When compound **27** was omitted from the reaction mixture, only trace amounts of radioactivity equivalent to background level were found in the 1-butanol extract, and a reduced amount of radioactivity was associated with the water layer. This indicated that the product formed had an affinity for organic solvent despite addition of sugar(s). The reaction was proportional with time up to 40 min (Figure 4A), and subsequent reactions were terminated after 20 min. Incorporation of radioactivity into the 1-butanol extract was also linear with respect to protein up to a concentration of 0.8 mg/ml. There was no significant increase of activity with increasing protein concentrations above this amount (Figure 4B). The reaction could be saturated with respect to the concentration of compound **27** at about 0.5 mM with a slight reduction in activity at 1 mM (Figure 4C). An apparent K_m of 2.3 mM can be calculated

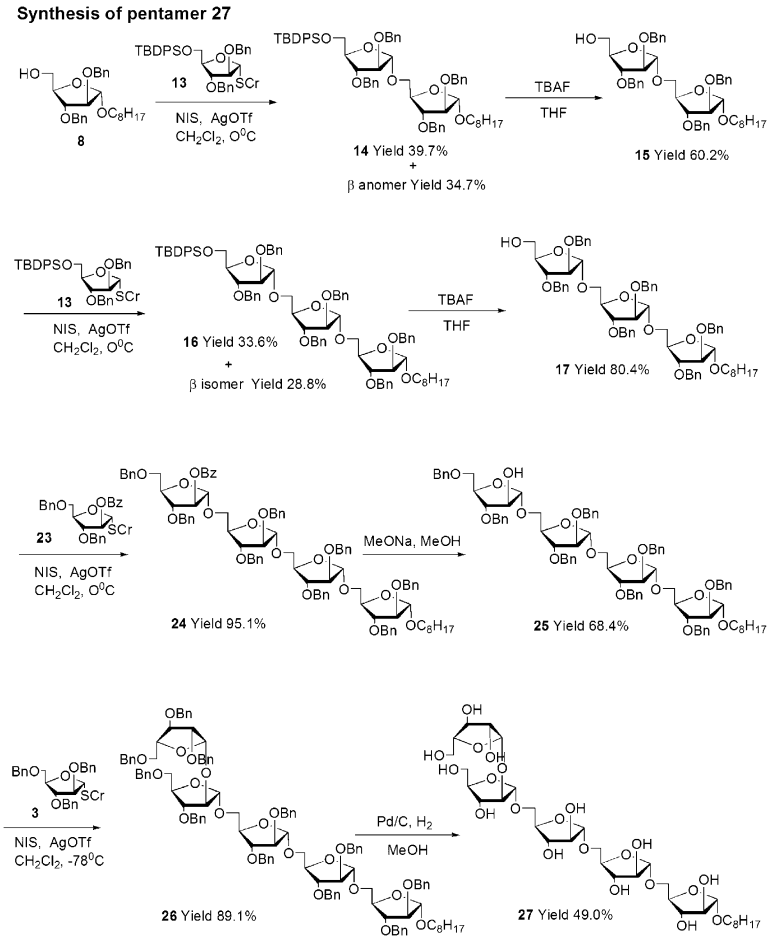

Figure 3. Synthesis of Pentamer **27**

Protected thiocresyl glycosides (Figure 2) were sequentially added to build the inner trimer (compound **17**) at the C-5 hydroxyl group. Compound **23** (Figure 2), carrying a benzoyl group at the 2-position, was then added. Debenzoylation followed by glycosylation with **3** at −78°C yielded the pentamer containing the terminal sugar in the β configuration. The reagents were added at the indicated intermediates of glycan synthesis.

from these data, assuming that there is substrate inhibition. This assumption is supported by the observation that at a concentration of 2 mM of compound **27**, the incorporation of radioactive material into the 1-butanol extract was reduced to 10% of maximal (data not shown). The concentration of the indirect donor of arabinose residues (p[^{14}C]Rpp) used was 3.8 μM and consequently the concentration of the direct arabinose donor (DPA) was probably much less. In addition, the endogenous synthesis of DPA relies on a number of enzyme activities and thus the concentration of this intermediate could not be efficiently manipulated. In a typical reaction, product was extracted with ethanol and excess p[^{14}C]Rpp was removed by passing through a strong anion exchange column (SAX) to remove p[^{14}C]Rpp. The ethanol eluate was dried and the residue was partitioned between water-saturated 1-butanol and water. All assays were performed in duplicates and typically afforded 4%–6% incorporation of radioactivity into the product. Thin-layer chromatography (TLC) analysis did not reveal any product formation when the enzyme source was used from either the *embA* or *embB* mutants. When equal amounts of membrane and P60 (based on protein estimation) from each of the *embA* and *embB* mutants were combined in the assay, a normal level of product formation was observed (Figure 5A). These data clearly indicate that the EmbA and EmbB proteins are responsible for arabinosyltransferase activity and that these proteins function together.

Characterization of the Arabinosyltransferase Reaction Product

The 1-butanol soluble material was analyzed by TLC and autoradiography, which revealed the presence of a single slower migrating new product in comparison to starting compound **27**, suggesting that the oligosaccharide had increased in size (Figure 5B). To confirm that [^{14}C]arabinose had been added to the acceptor, an aliquot of the 1-butanol-soluble material (~5000 dpm) was hydrolyzed with 2 M TFA and the hydrolysate was subjected to TLC. Autoradiography showed that the radioactivity was exclusively associated with arabinose (Figure 5C).

However, neither the mobility of the de novo synthesized neoglycolipid nor the sugars liberated after complete hydrolysis indicated whether there were one or two arabinosyl residues incorporated on the pentamer **27**, or the nature of the linkage. If one Ara*f* residue were incorporated, the product formed would be a hexamer, and would be resistant to *C. gelida* endoarabinanase digestion. In contrast, if two residues of Ara*f* were incorporated, the product would be a heptamer which would be amenable to the endoarabinanase digestion [16] leading to the formation of well-characterized Ara_6 (Figure 6B, peak 2) as was liberated from [^{14}C]Glc-labeled AG [19].

When the de novo synthesized product was subjected to endoarabinanase digestion followed by Dionex high pH anion exchange chromatography (HPAEC) using the radioactive detector, the digested material eluted

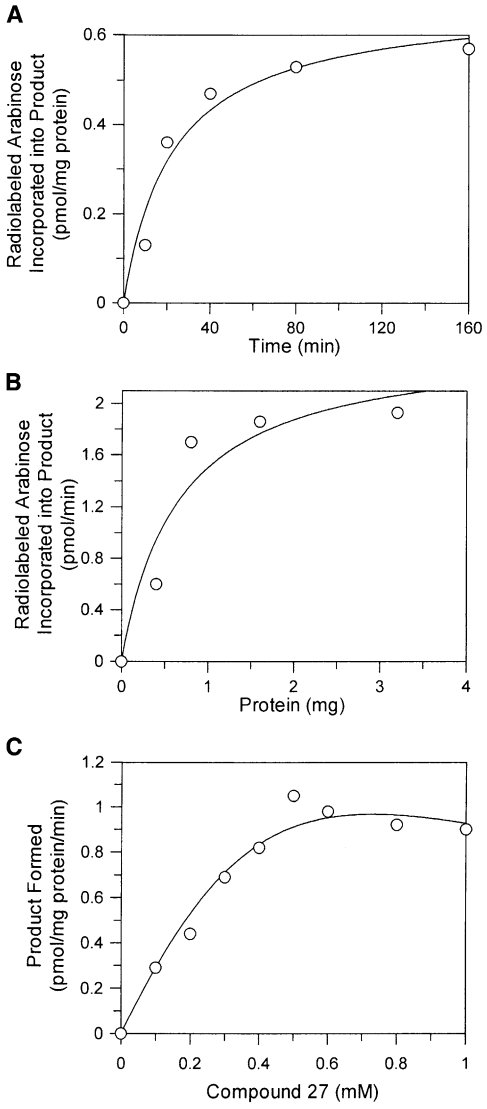

Figure 4. Time-Dependent Incorporation of Radioactivity in the Enzymatic Product and Effect of Protein and Acceptor Concentrations on the Rate of Product Formation

(A) Incorporation of radiolabeled [^{14}C]arabinose into product relative to time. At the indicated time points, the reactions were terminated by the addition of ethanol (200 μl), the supernatant was subjected to SAX columns, and extractions were counted for radioactivity. Concentration of the product formed was calculated from the counts (in dpm).

(B) Incorporation of radioactivity into the product relative to protein concentration. Four sets of reactions were performed, each having different protein (membrane + P60) concentrations (0.4, 0.8, 1.6, and 3.2 mg). The ratio of membrane to P60 was maintained as in the basic arabinosyltransferase assay. All other reaction conditions were identical to the basic arabinosyltransferase assay.

(C) Assays were performed at nine different substrate concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, and 2 mM), keeping all other reaction conditions similar. Control experiments without addition of any acceptor were also performed in parallel. The data obtained were subjected to nonlinear regression analysis using GraFit 5.0.

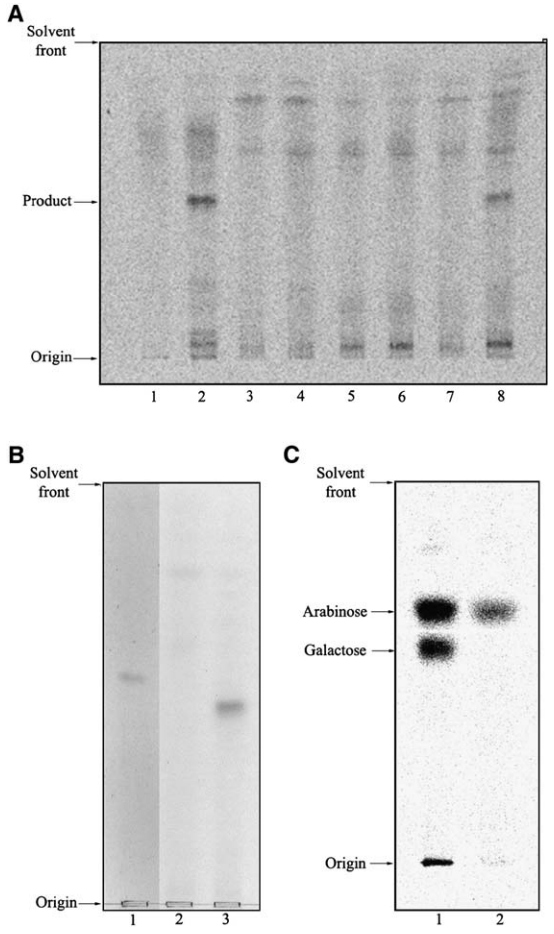

Figure 5. TLC Analysis of Pentamer **27** and the De Novo Product

(A) The reactions were set as *M. smegmatis* wild-type (lanes 1 and 2); *embA* mutant (lanes 3 and 4); *embB* mutant (lanes 5 and 6); and *embA* mutant + *embB* mutant (lanes 7 and 8). Lanes 1, 3, 5, and 7 are reactions without the addition of acceptor, and lanes 2, 4, 6, and 8 are reactions with acceptor. Lane 7 and 8 reactions contained equal amounts of protein membrane (0.25 mg each) and P60 (0.15 mg each) from *embA* and *embB* mutants. A 500 dpm aliquot of the product labeled with p[^{14}C]Rpp from the eluate after the SAX column followed by 1-butanol extraction (Experimental Procedures) was applied to a TLC plate developed in $CHCl_3$:MeOH:1 M NH_4OAc:NH_4OH:H_2O (180:140:9:9:23) and then exposed for 10 days.

(B) The starting material **27** was visualized after spraying with α-naphthol (lane 1); the radioactive product without (lane 2) or with (lane 3) acceptor was revealed by autoradiography. A 1000 dpm aliquot of the product labeled with p[^{14}C]Rpp from the ethanol eluate after the SAX column followed by 1-butanol extraction (Experimental Procedures) was applied to a TLC plate developed in $CHCl_3$:MeOH:1 M NH_4OAc:NH_4OH:H_2O (180:140:9:9:23) and then exposed for 4 days. The migration of the enzymatically synthesized product was slower than pentamer **27**.

(C) Radioactive AG (5000 dpm; lane 1) and the de novo product (lane 2) were hydrolyzed with 2 M TFA, the acid removed by evaporation, reconstituted in 25 μl of water, and applied to TLC. The chromatogram was developed in pyridine:ethyl acetate:acetic acid:water (5:5:1:3) and subjected to autoradiography.

with the same retention time as authentic Ara_6 (Figure 6B, peak 2) released from radiolabeled AG (Figure 6A). No Ara_2 was obtained from the endoarabinanase digestion of the de novo product.

Further confirmation of the characteristics of the endoarabinanase liberated radiolabeled product was sought from TLC/autoradiography. Unlabeled AG from *M. smegmatis* was digested with endoarabinanase, and the digest was subjected to HPAEC using a

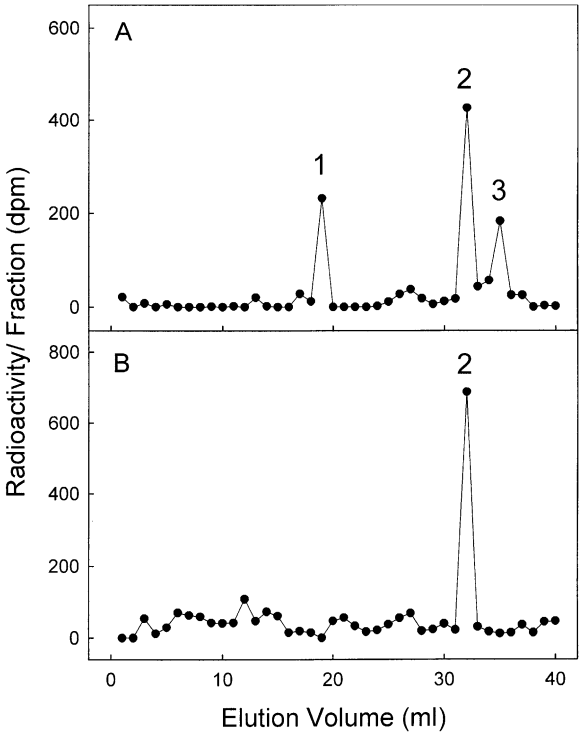

Figure 6. HPLC Analysis of Ara_6

(A) Base-solubilized [^{14}C]AG, 10,000 dpm from *M. smegmatis* mc^{2}155 cells [33], was digested with endoarabinanase as described, dried, reconstituted in 10 μl of water, and subjected to HPLC fitted with a Dionex Carbopac PA-1 column and a radioactive detector. Oligosaccharides were eluted with a gradient of sodium acetate (0–1 M) in 10% sodium hydroxide. Peaks labeled 1, 2, and 3 are Ara_2, Ara_6, and cyclic Gal_4, respectively. Identities of these peaks have been established earlier [37].

(B) Analogous conditions as for labeled AG were applied to the enzymatically synthesized product.

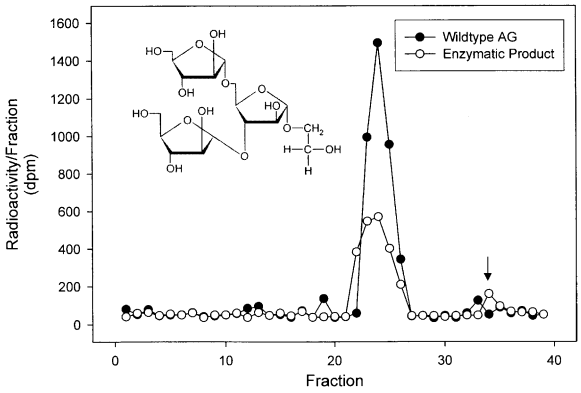

Figure 7. HPLC Analysis of Periodate Oxidation of Products

Radiolabeled Ara_6 from AG and Ara_6 released from the de novo product were isolated and subjected to a Smith degradation as described in Experimental Procedures. The samples were then analyzed by HPLC as in Figure 6. The inset shows the structure of the compound formed after Smith degradation. The down arrow at ~35 ml corresponds to residual Ara_6.

preparative column. The Ara_6 liberated was collected and desalted with an on-column carbohydrate membrane desalter (Dionex), and residual salt was removed using a Biogel P-2 column. Ara_6 released from the de novo product was analogously purified and both were applied to the TLC/autoradiography. The cold Ara_6 (confirmed using mass spectrometry) [16] and the labeled Ara_6 material comigrated, suggesting that they were the same compounds (data not shown).

This conclusion was further substantiated by periodate oxidation/Smith degradation. This procedure [20] should remove the two t-Ara*f* residues from the branched Ara_6, convert the 5-linked reducing arabinose to ethylene glycol, and yield a smaller oligosaccharide core from the remaining Ara*f* residues, the structure of which is shown in Figure 7 (inset). When Ara_6 isolated from AG that had been radiolabeled in vivo and the enzymatically synthesized product were subjected to Smith degradation, the products formed coeluted and had a retention time of 32 min (Figure 7), earlier than that of Ara_6 on HPAEC.

Discussion

The central role of arabinan in the cell wall proper appears to be in maintaining its integrity by tethering the parallel packed outer mycolic acid lipid barrier perpendicular to the underlying peptidoglycan through the flexible glycosyl linkages of AG. The ability of mycobacteria to synthesize AG is critical to its viability. Based on the linkages present and arrangement of various glycoside residues, one can speculate that a number (perhaps six or seven) of arabinosyltransferases are involved in the assembly of the arabinan chain(s) of mycobacterial AG [21]. However, only three proteins (EmbA, EmbB, and EmbC) have been directly implicated in arabinan synthesis in mycobacteria [14, 15, 22]. Only recently, a fourth arabinosyltransferase, AftA, has been identified and shown to donate the first arabinose to the galactan backbone in *Corynebacterium glutamicum* [23]. Unlike *Mycobacterium* species, *C. glutamicum* has only one *emb* gene which is reported to be able to donate [24] multiarabinosyl residues with different linkages.

It had been suggested that these proteins transfer the D-Ara*f* residues from DPA onto the growing arabinan [14]. Based on detailed structural studies on LAM and AG from the *embA*/*embB* mutants of *M. smegmatis*, we have previously shown that EmbA and EmbB are involved in the synthesis of the disaccharide β-D-(1→2)-α-D-Ara*f* on the 3-arm of the linear Araβ1→2Araα1→5Araα1→5Araα1→ in *M. smegmatis* [15]. Although the structural data were sound, we lacked the ability to assay for the putative activity of EmbA/EmbB.

In order to develop an arabinosyltransferase assay and to pinpoint the functional aspect of EmbA and EmbB, we synthesized an arabinose-based acceptor. The formation of oligosaccharide in high yield [25–27] and its use in the development of a mycobacterial arabinosyltransferase cell-free assay have previously been described [28], and there have been a number of elegant studies in recent years that describe efficient syntheses of furanosidic oligosaccharides related to mycobacterial cell wall arabinan leading to formation of oligosaccharides in high yield [18, 29–31]. To assess the role of the EmbA and EmbB proteins in the transfer of the disaccharide β-D-Ara*f*-(1→2)-α-D-Ara*f* intermediate involved in the formation of the terminal Ara_6, in the

present study, we synthesized a novel site-specific acceptor with the structure shown schematically in Figure 3, compound **27**, preserving the specific linkages and anomeric configurations. In designing the synthetic route to the target compound, we adapted an approach that could readily give a series of building blocks that could be assembled sequentially in order to provide the target linear pentamer **27**. With the building blocks in hand, all glycosylation reactions were carried out with an appropriate thioglycoside donor and octyl glycoside acceptor in the presence of *N*-iodosuccinimide and silver triflate [32]. The stereoselectivity of synthesis was determined by ^{13}C and ^{1}H NMR. Typically, the α-anomers resonated between δ109 and 106 ppm, whereas the β-anomer appeared at δ100.8 ppm. The main problem encountered during the course of chemical synthesis was that a mixture of α:β anomers was obtained in the synthesis of compounds **14** and **16** which required tedious chromatographic separation. This is due to the presence of a benzyl group at the 2-OH position of the donor. However, we maintained our strategy, as only a one-step deprotection would be required after assembly of the fully protected pentasaccharide. We also noticed that trace amounts of thiocresol remaining after purification of compounds **4** and **26** killed the Pd/C catalyst required for debenzylation, leading to low yields of **5** and **27**.

The molecule was designed such that it would serve as an acceptor substrate for the addition of Ara*f* residues by specific arabinosyltransferases, allowing us to probe the question of whether EmbA or EmbB was directly involved in the terminal Ara_6 formation. The enzyme activity assays and product analyses clearly demonstrated that EmbA and EmbB were involved in the transfer of two Ara*f* residues to the acceptor **27** in the cell-free system. However, the knockout mutants had detrimental effects; that is, the addition of Ara*f* residues to compound **27** was not detected by TLC analysis. This closely mirrored the in vivo phenotype of the arabinan determined in the EmbA and EmbB disrupted mutants [15]. The fact that neither the individual *embA* nor *embB* mutants could arabinosylate the acceptor efficiently and, more importantly, that arabinosylation was restored as in the wild-type upon mixing the enzyme sources from two individual strains, suggests that both proteins must be present for detectable activity in our assay. Therefore, it seems unlikely that either EmbA or EmbB protein by itself is an Ara*f* transferase. It is possible that the two proteins act in coordination to add the first Ara*f* residue to compound **27** and that a third protein (yet to be identified) adds the second residue.

Another hypothesis is that the terminal β-D-Ara*f*-(1→2)-α-D-Ara*f* disaccharide is presynthesized by "helper" enzymes and transferred in its entirety by the EmbB/EmbA functioning as a heterodimer. It is also possible that the Emb proteins are multifunctional and assemble, transfer, and/or transport the disaccharide. There could be multiple intermediate steps prior to the formation of β-D-Ara*f*-(1→2)-α-D-Ara*f* and then only the transfer will occur, for instance, (1) synthesis of the unique β-D-Ara*f*, (2) transfer of the β-D-Ara*f* to the 2-position of an α-D-Ara*f*, and (3) transfer of the disaccharide onto the 3-arm of the 3,5-α-D-Ara*f*. The role of EmbA and EmbB in mycobacteria is distinct from the role of the single Emb protein (Cg-emb) in *Corynebacterium* even though they share high homology [24]. Cg-emb is responsible for deposition of the majority of arabinan in AG (all but three residues) in *Corynebacterium*. It seems unlikely that if both EmbA and EmbB were absent a similar lack of arabinosylation of AG would occur.

Attributing functional properties to the Emb proteins will require synthesis of various specific neoglycolipid Ara*f* acceptors and continued analyses for acceptor activity. Attempts are also being made to generate double-knockout strains in *M. smegmatis*, such that functionality of these proteins can be addressed with a cleaner background.

Significance

In this study, we demonstrate unambiguously that a mycobacterial cell-free preparation from wild-type *M. smegmatis* transfers two Ara*f* residues from the donor pR[^{14}C]pp to the synthetic substrate, forming a nonreducing terminal disaccharide, β-D-Ara*f*-(1→2)-α-D-Ara*f* characteristic of the mycobacterial arabinan of AG. This transferase activity is not detectable in strains of *M. smegmatis* where either the *embA* or *embB* gene has been genetically disrupted, and is restored upon mixing the enzyme source from the two disrupted strains. These findings suggest that both EmbA and EmbB are required in the synthesis/transfer of the disaccharide to yield the terminal Ara_6 motif of AG.

This is the first description providing evidence for the role of the EmbB/EmbA protein in the formation of the crucial terminal Ara_6 in AG. These two proteins in a way contribute to the transfer and completion of the disaccharide β-D-Ara*f*-(1→2)-α-D-Ara*f* on a synthetic linear pentasaccharide. The results are in complete agreement with the phenotypic changes observed for EmbA and EmbB deleted mutants. The mycobacterial arabinosyltransferases are unique and attractive targets for developing new antimicrobial therapeutics. Our work paves the way for probing for numerous arabinosyltransferases that should logically be present in the assembly of complex molecules such as arabinan of LAM and AG.

Experimental Procedures

A preparation of p[^{14}C]Rpp (300 mCi/mmol) was generated from uniformly labeled D-[^{14}C]glucose (American Radiolabeled Chemicals) as described [10]. ATP, MOPS, 2-mercaptoethanol, DNase (type I), and RNase (type I) were purchased from Sigma. Percoll (sterile) was purchased from Pharmacia. Biogel P-6 and P-2 were obtained from Pharmacia. Authentic pure Ara_6 was obtained after enzyme digestion of AG followed by collecting the relevant peak eluting off Dionex HPAEC and immediate desalting by passage through a Biogel P-2 column. Radiolabeled Ara_6 was isolated from radiolabeled AG purified from *M. smegmatis* that was metabolically labeled as previously described [33]. Protein concentrations were determined using the BCA assay (Pierce). All compounds synthesized are distinguished in bold type font.

Organic Synthesis and Analysis

All chemicals were purchased from Sigma-Aldrich and were used without further purification. Thin-layer chromatography (TLC) was performed on silica gel G 60 (EM Science) aluminum-backed plates. Column chromatography was performed using silica gel (70–230 mesh) and Iatrobeads (beaded silica gel 6RS8060) (Iatron

Laboratories, Tokyo). Unless otherwise stated, all reactions were performed at room temperature under argon. Drying after organic extractions was done over anhydrous Na_2SO_4.

^1H and ^{13}C NMR spectra for the synthetic compounds were measured on a Varian-Inova 300 or 400 spectrometer. Electrospray (ESI) and fast atom bombardment mass spectrometry (FAB-MS) were performed on Thermo-Finnigan LCQ Duo and VG Autospec mass spectrometers, respectively. High pH anion exchange chromatography (HPAEC) was performed on a Dionex LC system fitted with a Dionex Carbopac PA-1 column, and the oligosaccharides were eluted using a gradient of 0–1 M sodium acetate in 10% sodium hydroxide and detected with a pulse-amperometric detector (PAD).

For chemical synthesis, donors and acceptors were synthesized independently as building blocks as shown in Figures 2 and 3. The two donors **13** (*p*-cresyl 2,3-di-*O*-benzyl-5-*O*-tertbutyldiphenylsilyl-1-thio-α-D-arabinofuranoside) and **23** (*p*-cresyl 2-*O*-benzoyl-3,5-di-*O*-benzyl-1-thio-α-D-arabinofuranoside and the acceptor **8** octyl 2,3-di-*O*-benzyl-α-D-arabinofuranoside were synthesized following documented procedures [28, 34, 35] with variations as needed. The inner trisaccharide **17** was built on the acceptor **8**, which served as a template for the addition of compound **23**, to yield tetramer **24** in 95% yield. Selective removal of the benzoyl group from the 2-OH position of **24** yielded an intermediate **25** suitable for β-glycosylation [35] to give the fully protected pentamer **26** in 89% yield: R_f 0.15 (hexane:EtOAc, 4:1); ^1H NMR (300 MHz, $CDCl_3$): δ7.37–7.21 (m, 55 H), 5.15 (s, 1 H), 5.13 (s, 1H), 5.11 (s, 2 H), 5.10 (s, 1 H), 4.81–4.37 (m, 22 H), 4.25–4.01 (m, 10 H), 3.94–3.82 (m, 4 H), 3.74–3.66 (m, 4 H), 3.58–3.52 (m, 8 H), 3.42–3.34 (m, 1 H), 1.60 (m, 2 H), 1.29 (m, 10 H), 0.90 (m, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ138.5, 138.4, 138.3, 138.0, 137.9, 137.8, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.0, 127.9, 127.8, 106.7, 105.3, 100.6, 88.7, 88.5, 85.9, 84.5, 84.2, 83.6, 83.4, 83.2, 81.8, 80.8, 80.2, 73.5, 73.3, 72.6, 72.5, 72.4, 72.2, 70.3, 69.0, 68.0, 66.2, 65.8, 32.1, 30.0, 29.7, 29.6, 26.4, 22.9, 14.4. FAB-MS calculated for $[C_{110}H_{124}O_{21}]$ 1782.15, found 1782.1.

Compound 27

Debenzylation in the presence of Pd/C gave the desired product (49%), which was found to be extremely water soluble. R_f 0.78 ($CHCl_3$:MeOH:H_2O, 10:10:3) ^1H NMR (400 MHz, D_2O): δ 5.14 (s, 1 H), 5.11 (d, 1 H, J = 3.6 Hz), 5.08 (s, 1 H), 5.05 (s, 1 H), 4.97 (s, 1 H), 4.16–3.95 (m, 15 H), 3.84–3.60 (m, 12 H), 1.57 (m, 2 H), 1.25 (m, 10 H), 0.85 (m, 3 H). FAB-MS calculated for $[C_{33}H_{58}O_{21}]Na^+$ 813.35, found 813.3.

Preparation of Mycobacterial Membranes and P60

Wild-type and *embB* and *embA* mutants of *M. smegmatis* mc^2155 cells were grown in nutrient broth (Difco Laboratories) to mid-log phase (OD 0.7–0.8). Cells (~8–10 g wet weight) were harvested by centrifugation, washed, and resuspended in ice-cold buffer A containing 50 mM MOPS (pH 8.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 150 μg/ml DNase, and 250 μg/ml RNase. Cells were disrupted by six to seven passages through a French press at 1500 psi. The suspension was centrifuged at 27,000 × g for 60 min at 4°C. The mycobacterial cell wall pellet was removed and the supernatant was recentrifuged at 200,000 × g for 2 hr at 4°C. The supernatant was discarded and the pellet of enzymatically active membranes was gently resuspended in 500 μl of buffer A; the resulting protein concentrations of the membrane fractions were typically between 15 and 20 mg/ml. The 27,000 × g pellet was suspended in 10 ml of buffer A and Percoll was added to achieve a 60% suspension. The suspension was mixed and centrifuged at 27,000 × g for 60 min at 4°C. The resulting flocculent, white layer was collected and washed three times with buffer A at 12,000 × g to yield a cell wall-enriched fraction (P60). This fraction was resuspended in 1 ml of buffer A to yield a protein concentration of 5–6 mg/ml.

Arabinosyltransferase Assays

Typical reaction mixtures contained 40 mM MOPS (pH 8.0), 4 mM 2-mercaptoethanol, 8.5 mM $MgCl_2$, 1 mM ATP, 3.8 μM p[^{14}C]Rpp (500,000 dpm), the indicated concentrations of acceptor (compound **27**), and membranes and P60 in a total volume of 200 μl. The reaction mixtures were incubated at 37°C for 1 hr and then terminated by adding 200 μl of ethanol. The resulting mixture was centrifuged at 14,000 × g, and the supernatants were loaded onto prepacked strong anion exchange (SAX) columns (Burdick and Jackson). The columns were eluted sequentially with 2 ml each of 50% ethanol and water. The eluate was evaporated to dryness and partitioned between the two phases (1:1) of water-saturated 1-butanol and water. The 1-butanol fractions were measured for radioactive incorporation by liquid scintillation spectrometry. Assays were carried out under conditions where synthesis was linear for both time and protein concentration. Values reported are averages of duplicate reactions from representative experiments. Kinetic constants were determined by nonlinear regression analysis using GraFit 5.0 (Erithacus Software).

Analytical Procedures

For TLC analysis of the enzymatically synthesized, radiolabeled product, an aliquot of the 1-butanol extract was dried under nitrogen and the residue was dissolved in methanol for analysis by silica gel TLC. The plates were developed in $CHCl_3$:MeOH:1 M NH_4OAc:NH_4OH:H_2O (180:140:9:9:23) followed by autoradiography at −70°C using Biomax MR1 film (Kodak), and compound **27** was visualized using α-naphthol spray reagent. For analysis of the sugar composition of the radiolabeled product, approximately 5000 dpm of the 1-butanol-soluble material was dried under nitrogen and hydrolyzed in 200 μl of 2 M trifluoroacetic acid (TFA) at 120°C for 2 hr. The TFA was removed under a stream of air, and the hydrolysate was analyzed on a silica gel TLC plate developed in pyridine:ethyl acetate:acetic acid:water (5:5:1:3) followed by autoradiography as described above. Radioactive spots were identified by cochromatography with standard sugars, which were visualized using α-naphthol spray.

Radiolabeled products from the enzyme assays were subjected to digestion with endoarabinanase isolated from *C. gelida* [16]. In this case, approximately 10,000 dpm of the 1-butanol-soluble material was dried under nitrogen, reconstituted in 10 μl of water, sonicated, and treated with endoarabinanase for 16 hr at 37°C as previously described [36]. Aliquots from the digested mixture containing the released oligoarabinofuranosides were subjected to Dionex HPAEC. Radioactivity was quantitated by liquid scintillation spectrometry.

The endoarabinanase-treated radioactive material was also subjected to Smith degradation (periodate oxidation) as previously described [16, 20]. Briefly, enzymatically radiolabeled, endoarabinanase-treated digestion product (10,000 dpm) was purified by size exclusion chromatography on Biogel P-6. This material and authentic Ara_6 isolated from endoarabinanase-digested radiolabeled AG were subjected to periodate oxidation (0.15 M) for 72 hr. Ethylene glycol was added to terminate the reaction. The oxidized product was desalted on a Biogel P-2 column and reduced with sodium borodeuteride. After removal of the borate after coevaporation with methanol, the residue was subjected to mild acid hydrolysis (1 N H_2SO_4) overnight at room temperature and the mixture was neutralized with barium carbonate. The reaction mixture was then centrifuged and the supernatant was dried, dissolved in water, desalted, and analyzed by Dionex HPAEC and TLC.

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