Galactosamine in walls of slow-growing mycobacteria

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Galactosamine was found consistently as a minor component of the envelope of five species of slow-growing mycobacteria, including all the major human pathogens, but not three rapid-growing species. The amino sugar was a component of the arabinogalactan of the cell wall skeleton, and occurred at the level of about one residue per arabinogalactan chain. Its amino group was in the free, un-N-acetylated state. Examination of oligosaccharides released by partial acid hydrolysis of arabinogalactan by fast atom bombardment-MS and gas chromatography–MS identified a series of oligoarabinans, each possessing one GalN unit, linked to position 2 of arabinose. It is proposed that the GalN residues occur as stub branches of $1 \rightarrow 5$-linked arabinose chains in the arabinogalactan. Possible functions of GalN are discussed.

INTRODUCTION

Galactosamine (2-amino-2-deoxygalactose; GalN) has been reported sporadically as a component of mycobacterial envelopes. Wax D of mycobacteria, an adjuvant-active glycolipid obtained from mycobacterial cells by solvent extraction of the wall, was reported to contain considerable amounts of GalN [1]. Wax D was subsequently recognized as a derivative, probably a degradation product, of the wall. However, the cell wall skeleton itself, the covalently linked component of the wall that remains when all solvent-extractable components are removed, contains, at most, minor quantities. In early reports [2,3], which depended on paper chromatography for the detection of wall components, GalN was not detected. However, with the greater sensitivity of the automatic amino acid analyser, GalN was measured in cell wall skeleton from *Mycobacterium lepraemurium* [4] and *M. microti* [5]. GalN has also been found in the wall of *M. avium* but not in the rapid-growing species *M. smegmatis* (M. Daffé, personal communication). GalN units do not appear in any of the published models of the structure of the cell wall skeleton and their situation in the structure has never been investigated.

The mycobacterial cell wall skeleton, as understood at present, consists of a layer of peptidoglycan differing from that of *Escherichia coli* only in the substitution of *N*-glycolyl groups for *N*-acetyl groups, to which are attached branched-chain arabinogalactan (AG) units esterified at their distal ends with long branched-chain fatty acids, mycolic acids [6]. This structure is common to slow-growing and rapid-growing species of mycobacterium, with a minor difference only in *M. leprae*. The slow-growing group includes all the important pathogenic species.

There is considerable current interest in the structure and biosynthesis of the mycobacterial wall because it contains several chemically unique features that offer excellent targets for novel antibiotics. Such new drugs are badly needed to help in the control of tuberculosis, a mycobacterial disease that remains a major public health problem and causes about three million deaths each year. An investigation into the location of GalN in the wall (or even a proof that it is really a covalent part of the structure) therefore seemed warranted. Most of our investigations have been with *M. microti*, a species closely related to *M. tuberculosis* although not itself a human pathogen, but we have also made limited investigations of four major human pathogens, *M. tuberculosis*, *M. leprae*, *M. kansasii* and *M. avium*, as well as the vaccine strain *M. bovis* BCG and some non-pathogenic rapid-growing species. We have found that GalN is a covalently bound component of the walls of slow-growing mycobacteria, and have confirmed that it does not occur in the corresponding situation of rapid-growing species. It is attached to an arabinose unit of AG of the cell wall skeleton, and occurs as the free amine rather than as the more usual *N*-acetyl compound.

MATERIALS AND METHODS

Strains and culture conditions

M. microti OV254 is maintained at the National Institute for Medical Research on Löwenstein–Jensen slopes. Cells were grown up at 37 °C in Tween®/glutamate medium ('glycerol-free medium' [7], modified from the glycerol-free medium of [8]), 3 litres in 5 litre conical flasks, stirred with a magnetic stirrer bar. After 4 to 5 days, when the attenuance at 450 nm had reached 0.7–0.9, the cells were harvested by centrifugation at 16000 ***g*** for 5 min, washed once with Tris/Tween® [0.1 M Tris/HCl (pH 7.2)/0.1 % Tween®-80], and resuspended in approx. 40 ml of Tris/Tween®.

M. kansasii 9059 was obtained from P. A. Jenkins (Mycobacterium Reference Laboratory, Cardiff, South Glam., U.K.) and grown for 3–4 weeks on slopes of 7H9 agar with OADC supplement (Difco) in 100 ml screw-capped plastic jars. The jars were irradiated with 500 krad (5 J/kg) from a ^{60}Co source for safety reasons, and the bacteria were then rinsed off the slopes with water, washed with water and freeze-dried.

Rapid-growing mycobacterial species *M. neoaurum* NCTC 10818 and *M. phlei* NCTC 8151 were obtained from the National Collection of Type Cultures, London; *M. vaccae* R877R (now

Abbreviations used: ABEE, ethyl 4-aminobenzoate; AG, arabinogalactan; FAB–MS, fast atom bombardment MS; Gal, galactosamine.

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NCTC 11659) was kindly provided by J. L. Stanford. Strains were grown in Lemco broth [9] at 37 °C for 2–4 days with shaking, collected and washed once with Tris/Tween®.

M. tuberculosis TMC107 (strain Erdman) was grown for 6–8 weeks on a glycerol/alanine/salts medium as described previously [10]. *M. bovis* BCG (Danish strain; Lilly) was obtained in freeze–dried form from S. D. Chaparas (Bureau of Biologics, Food and Drug Administration, Bethesda, MD, U.S.A.).

M. avium serovar 4 was grown at 37 °C in 7H11 broth, 2 litres in 4 litre conical flasks, with shaking. Cells were heat-killed isothermally at 80 °C for 1 h and were harvested by centrifugation at 5000 ***g*** for 10 min, washed once with PBS and stored frozen.

Preparation and purification of walls

Suspensions of *M. microti* were sonicated at 90 W with a 12.5 mm diameter probe 20 times (1 min each), with 1 min intermissions for cooling. A stainless steel vessel surrounded by ice/water was used to promote good cooling. In a single large-scale experiment *M. microti* grown in a 50 litre fermenter in Tween®/glutamate for 4.5 days (D_{450} 1.7) was washed with Tris/Tween® and broken in a Manton–Gourlin press. Suspensions of broken cells in Tris/Tween® were digested with DNAase and RNAase (approx. 100 μg/ml) in the presence of sodium azide (0.02 %) and 1 mM Mg^{2+} at 4 °C for 16 h, then adjusted to 40 % (v/v) Percoll® (Pharmacia) and centrifuged for 10 min at 27000 ***g***, followed by 1 or 2 h at 12000 ***g***, in an angle rotor. Short liquid columns, with a height about twice the diameter, were used to obtain consistent gradient formation. The wall band was removed, washed three times with Tris/Tween®, heated at 100 °C for 10 min in 1 % (w/v) SDS, washed once with Tris/Tween®, once with 1 M NaCl, twice with Tris/Tween®, once with 0.1 % Tween®-80 in 1 mM Mes, pH 5, once with water and then freeze-dried.

Walls of rapid-growing species were prepared similarly, except that they were sonicated 15 times for 1 min each. Walls of *M. tuberculosis* Erdman, *M. bovis* BCG and *M. avium* were prepared as described previously [11].

Suspensions of *M. leprae* in PBS were sonicated at 1.5 MHz at 50 % duty cycle (1 s pulse intervals) six times for 5 min each, with 5 min intermissions for cooling. The suspension was twice centrifuged at 27000 ***g*** for 30 min at 4 °C. The pellet was then extracted three times with 2 % (w/v) SDS in PBS at 56 °C for 1 h, followed by 0.5 % SDS in PBS containing 2 mg/ml of proteinase K (1 ml of buffer per 0.1 g of cells) at 37 °C with stirring for 12–16 h, and finally with 0.5 % SDS in PBS containing 2 mg/ml of Pronase® at 37 °C for 16 h. The protein-free pellet was then washed once with 2 % SDS in PBS and three times with PBS, followed by a final extraction with acetone at room temperature.

Preparation of AG

Freeze-dried walls (*M. microti*, *M. bovis* BCG, *M. avium* and the rapid-growing species) or freeze-dried whole cells (*M. kansasii*) were freed from lipids, and the AG was released with dilute acid as described previously [12], except that a preliminary extraction step with ethanol/diethyl ether (1:1, v/v) was included and the acid treatment was shortened to 4 days. AG from *M. kansasii*, which was expected to be contaminated with mycobacterial proteins because it was prepared from whole cells, was further purified by treatment with hot 44 % (w/v) phenol [13] followed by dialysis to remove the phenol.

AG of *M. microti* was also prepared by heating mycolate-free walls with 1 M NaOH at 75 °C for 8 h with occasional stirring. The digest was cooled with ice, neutralized with acetic acid and centrifuged at 23000 ***g*** for 20 min. The clear supernatant was dialysed against water, evaporated to a small volume, partitioned with 44 % (w/v) phenol and further dialysed. AG was base-solubilized from *M. tuberculosis* Erdman and *M. avium*, as described previously [11].

Fractionation of AG

AG was eluted from 26 mm × 200 mm or 26 mm × 600 mm columns of Biogel® P-10 (Bio-Rad) with either HPLC-grade water (Romil) or 0.05 M NaCl at approx. 20 ml/h. Fractions were pooled on the basis of neutral sugar and amino group content, then evaporated; pools containing 0.05 M NaCl were first dialysed against water. The columns were calibrated with carbonic anhydrase (excluded), cytochrome *c*, vitamin B_{12} and galactose (all from Sigma). Proteins (but not carbohydrates) could be eluted successfully only with 0.05 M NaCl.

Partial hydrolysis and separation of oligosaccharides

Whole AG or fractions from Biogel® P-10 were heated at 60 °C in 0.5 M H_2SO_4 for 3 h, neutralized by stirring with $BaCO_3$, filtered and evaporated. Partial hydrolysates applied in water were eluted from a 26 mm × 600 mm Biogel® P-2 column with 0.05 M NaCl in HPLC-grade water at approx. 20 ml/h. Fractions were pooled on the basis of amino group content and evaporated. The column was calibrated with cytochrome *c* (as an excluded substance) and glucose, maltose and malto-oligosaccharides up to maltoheptose (Sigma).

Chemical processing of GalN-containing carbohydrates

N-Acetylamino sugars were deacetylated with sodium hydroxide in DMSO [14]. Amino sugars with a free amino group were destroyed with nitrous acid [15]. Free amino groups were acetylated by treatment for 30 min at room temperature with a mixture of 100 μl of methanol, 50 μl of pyridine and 50 μl of acetic anhydride and N-deuteroacetylated by standing the sample in a mixture of 500 μl of methanol, 50 μl of d_6-acetic anhydride (Aldrich) and 10 μl of pyridine for 15 min at room temperature. The free amine and all other free hydroxy groups were also directly perdeuteroacetylated by incubation of the sample in pyridine/d_6-acetic anhydride (1:1, v/v) at 80 °C for 2 h. The high concentration of salt present in oligosaccharide samples was removed by repeated chloroform/water partitioning after per-deuteroacetylation. Permethylation of the perdeuteroacetylated derivatives was performed with the NaOH slurry method [16]. N-deuteroacetylated oligosaccharides were tagged with ethyl 4-aminobenzoate (ABEE) by dissolving the sample in 10 μl of water, followed by the addition of 50 μl of the derivatizing reagent (consisting of 350 μl of methanol, 41 μl of acetic acid, 33 mg of ABEE and 10 mg of sodium cyanoborohydride) and left at 80 °C for 1 h. Excess reagents were removed by redissolving the dried sample in water and washing repeatedly with ether. Salts were further removed by reverse-phase HPLC with a Vydac® C_{18} column (Rainin; 0–40 % acetonitrile in 40 min, monitored by absorbance at 304 nm).

Analytical techniques

Neutral sugars were estimated with the phenol/sulphuric acid method [17] with galactose as a standard. Amino groups were assayed with the fluorescamine reagent [18], with glucosamine/HCl as standard; fluorescence was measured in a Shimadzu RF-540 spectrofluorimeter. Fluorescamine was obtained from Sigma. Amino sugars and amino acids were determined with an automatic amino acid analyser after hydrolysis either with 4 M HCl at 105 °C for 16 h or with 3 M toluene-*p*-sulphonic

acid at 100 °C for 24 h [19]. The identity of the non-glucosamine amino sugar was determined by hydrolysing the polymers (both intact cell wall and base-solubilized AG) with 4 M HCl for 4 h at 100 °C and converting sugars to alditol acetates after N-acetylation. Alditol acetates were co-injected with standard GalNAc on a DB-1 GC column operated at an initial temperature of 100 °C for 4 min followed by a temperature gradient of 8 °C/min to a final temperature of 280 °C.

Fast atom bombardment–MS (FAB–MS) analysis

FAB mass spectra were obtained with a VG Analytical ZAB-2SE 2FPD or an Autospec mass spectrometer fitted with a caesium ion gun operated at 25–30 kV. Data acquisition and processing were performed with the VG Analytical Opus software. Monothioglycerol was used as matrix, and derivatized samples were dissolved in methanol.

RESULTS

Hexosamine content of AG of *M. microti*

AG released from delipidated walls of *M. microti* by dilute acid was assayed for neutral sugars; there was good agreement between the mass of the samples and the carbohydrate content, indicating that the material was essentially pure polysaccharide. The material contained both glucosamine (GlcN) and GalN, as determined by amino acid analysis. Amounts varied somewhat in different preparations, but were typically about 60 nmol of GalN and a slightly smaller amount of GlcN per mg of neutral sugar. Small amounts of amino acids were present, mostly derived from traces of peptidoglycan.

It was at first assumed that the GalN would be present as its *N*-acetyl derivative, and an attempt was made to deacetylate it to exploit the properties of the free amino group for specific labelling of the molecule and for selective hydrolysis. Removal of the acetyl group was monitored with the fluorescamine reagent. However, it was found that the deacetylation reaction had no effect on fluorescamine reactivity, although this reactivity was removed by N-acetylation. Further, treatment of the AG with nitrous acid destroyed all the GalN detectable by amino acid analysis unless the material had first been N-acetylated. Treatment of *N*-acetylglucosamine (Sigma) with dilute H_2SO_4 under the conditions used to release AG from lipid-free walls resulted in the formation of little (less than 5%) free GlcN, measured by the formation of fluorescamine-reacting material. It was concluded that the amino group of the GalN in the native AG was not acetylated.

The progress of solubilization of hexosamines from lipid-free walls by 0.05 M H_2SO_4 was followed by sampling the hydrolysis mixture at intervals and removing insoluble material from the samples by centrifugation. The release of GalN closely paralleled the release of neutral sugar (representing the hydrolysis of the linker joining the AG to the peptidoglycan), and reached a plateau corresponding to the total GalN content of the material after 5 days. The release of GlcN was relatively delayed, but continued in a roughly linear fashion throughout the hydrolysis. It seemed likely that this reflected the partial hydrolysis of the peptidoglycan portion of the wall in addition to the release of GlcN involved in the linker unit between the peptidoglycan and the AG.

Fractionation of AG with Biogel® P-10

The material released by treatment of AG with dilute acid was heterodisperse and could be separated into several fractions by gel filtration (Figure 1). The pattern obtained was reproducible between preparations. The apparent molecular mass of the first (major) peak was slightly greater than that of cytochrome *c*. The peaks of carbohydrate (neutral sugar) coincided with peaks of fluorescamine-reactive material except in the case of the low-molecular-mass peak, which consisted mainly of monosaccharide, as determined by TLC, but contained no fluorescamine-reactive material. For comparison, a batch of AG released from lipid-free walls of *M. microti* with sodium hydroxide was prepared and fractionated with Biogel® P-10 (results not shown). The starting material had been dialysed to remove sodium acetate, so it was not possible to determine whether the original sample contained monosaccharide, but it was otherwise apparently monodisperse and the single peak of material had a higher apparent molecular mass than any of the materials released by acid (approximately coinciding with the elution position of carbonic anhydrase). This confirms that some degradation of AG occurred during its release by dilute acid. Alkali-released AG was contaminated with large amounts of fluorescamine-reactive polymers with a broad distribution of apparent molecular mass, mostly smaller than the main carbohydrate-containing peak. Amino acid analysis of hydrolysed fractions showed that the contaminant was mainly glutamic acid, presumably derived from polyglutamic acid present in the original walls. GalN was present in the high-molecular-mass fraction, which also contained almost all the neutral sugar.

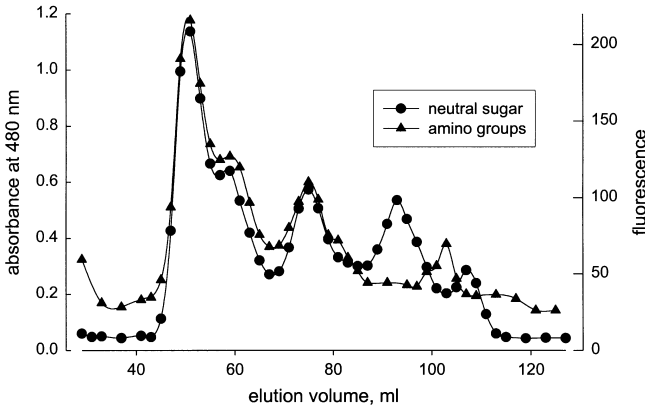


Figure 1 Elution pattern of acid-solubilized AG of *M. microti* from a 26 mm × 200 mm column of Biogel® P-10, with water as eluent

Fractions were assayed for neutral sugars and amino groups (see the Materials and methods section). Cytochrome *c* was eluted at approx. 55 ml from this column; galactose was eluted at approx. 105 ml.

Confirmation of identity of GalN

Although the amino acid analyser detected two species of amino sugar, of which one was certainly GlcN, the identification of the second peak as GalN was not unambiguous. The identity of GalN was confirmed by releasing the substance from walls and AG with 4 M HCl, converting it to its *N*-acetyl alditol acetate and examining it by GC along with standard GalNAc, with which it was co-eluted.

Isolation of GalN-containing oligosaccharides

Advantage was taken of the fact that the strong positive charge on the amino group of GalN could protect nearby glycosidic linkages from hydrolysis by acid. Conditions were chosen, on the

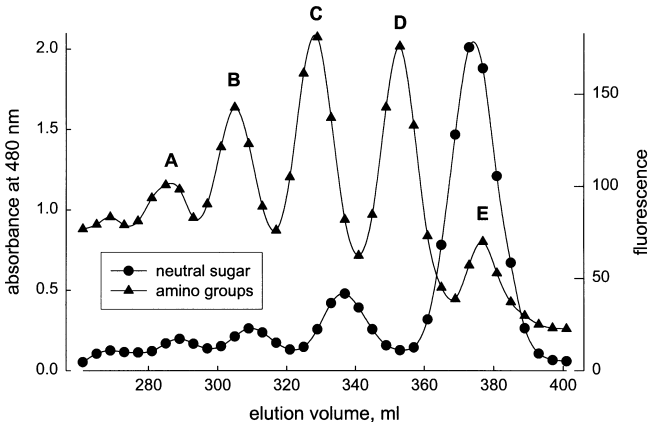


Figure 2 Elution pattern of partly hydrolysed high-molecular-mass peak of AG (Figure 1) from a 26 mm × 600 mm column of Biogel® P-2

Fractions were assayed for neutral sugars and amino groups, then pooled as indicated to provide the materials for the analyses shown in Table 1.

Table 1 Oligosaccharide fractions from partly hydrolysed AG of *M. microti*

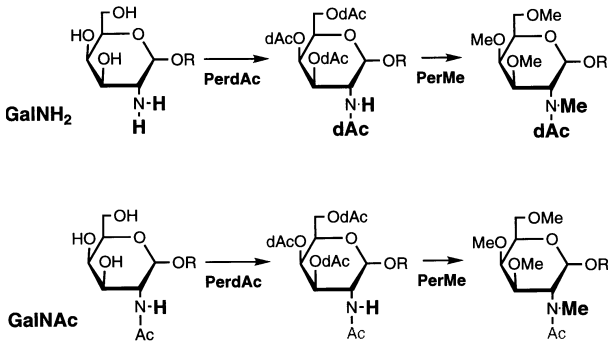
Material with the highest apparent molecular mass that was eluted from Biogel® P-10 (Figure 1) was partly hydrolysed and further separated with Biogel® P-2 (Figure 2). Fractions were pooled as indicated and analysed for free amino groups with fluorescamine and for oligosaccharides by FAB–MS analysis of their chemical derivatives.

Fraction	Amino groups (nmol in sample)	Oligosaccharides		
		Gal_n	Ara_n	GalN-Ara_n
A	89	Gal_4	Ara_4	GalN-Ara_5
B	127	Gal_3	Ara_4	GalN-Ara_4
C	216	Gal_2	Ara_2	GalN-Ara_3
D	199	Gal, Gal_2	Ara_2	GalN-Ara_2
E	42	Gal	Ara	

basis of preliminary experiments, such that almost all the AG was hydrolysed to monosaccharide. Fractionation on a column of Biogel® P-2 and analysis of the fractions for neutral sugar and fluorescamine-reactive material produced the pattern shown in Figure 2. The bulk of the carbohydrate was, as expected, present as arabinose and galactose, which were not separated on this column, although the very broad shape of the monosaccharide peak suggests that the peaks of the two monosaccharides were not coincident. Small amounts of higher oligosaccharides were present; their sizes were estimated by comparison with oligosaccharide standards. A more extended series of fluorescamine-reactive peaks was also present that did not fully coincide with the neutral oligosaccharide peaks or with the positions of the standard oligosaccharides. None of these peaks contained free hexosamine, which elutes later than galactose from P-2. Fractions corresponding to the peaks of fluorescamine-reactive material were pooled, evaporated, redissolved in small volumes of water and subjected to further chemical and mass spectrometric analysis (Table 1).

Identification of galactosaminyl arabinans

Although the only neutral sugars present in AG and its partial hydrolysates were Gal and Ara, the exact chemical identity of the



Scheme 1 Perdeuteroacetylation and permethylation of GalN and GalNAc

Perdeuteroacetylation followed by permethylation yields volatile derivatives with different masses according to whether GalN or its *N*-acetyl derivative was the starting material; these can be distinguished by MS.

oligosaccharide components present in each pooled fraction was unknown, particularly with respect to the fluorescamine-reactive material. To provide unequivocal evidence for the presence of GalN-containing oligosaccharides, the pooled fractions were first screened by FAB–MS after chemical derivatization. Conditions were chosen under which GalN-containing oligomers could be distinguished from GalNAc-containing ones by virtue of their mass difference after perdeuteroacetylation and permethylation, as shown in Scheme 1.

It was found that one of the pooled fractions contained only galactose and arabinose; the others each contained three major components, namely a Gal_n oligomer, an Ara_n oligomer, and another product that could be assigned as a GalN-containing Ara_n oligomer on the basis of its distinctive mass values after derivatization. The respective components identified in each fraction are tabulated in Table 1. For example, fraction C after perdeuteroacetylation afforded major signals at m/z 575, 725, and 1093 in its FAB mass spectrum, which corresponded respectively to sodiated molecular ions of Ara_2, Gal_2 and $Ara_3GalNHdAc_1$. Further permethylation converted the perdeuteroacetylated $Ara_3GalNHdAc$ product into permethylated $Ara_3GalNdAcMe$, which gave a sodiated molecular ion at m/z 775, as well as a strong oxonium fragment ion signal at m/z 263, corresponding to $GalNdAcMe^+$. The mass data, together with the fluorescamine-positive assay, firmly established that a series of GalN-containing arabinan oligomers, and not GalNAc-containing arabinans, were being produced by partial hydrolysis. The presence of the non-reducing terminal ion $GalNdAcMe^+$ further indicated that the GalN residue occurred as a terminal residue covalently linked to the arabinan chain. This is confirmed by gas chromatography–MS linkage analysis of the permethylated products, in which terminal GalNAc was identified as the only detectable alditol acetate containing HexNAc (where Hex stands for hexose) (results not shown).

Further characterization of GalN-Ara_n

It should be noted that although the GalN-Ara_n products could be readily identified in the GalN-enriched hydrolysates, they nevertheless constituted only minor components relative to the incompletely hydrolysed oligomers of Ara and Gal (Table 1). To facilitate removal of sodium chloride introduced during the Biogel® P-2 chromatography and further characterization of the GalN-Ara_n product, the free reducing end of each of the oligosaccharide components present in fraction D was tagged

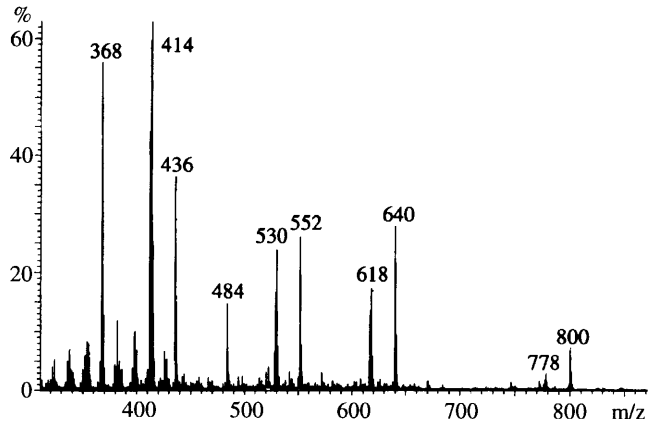


Figure 3 FAB mass spectrum of permethylated, N-deuteroacetylated ABEE derivatives of fraction D of Figure 2

In addition to the sodiated molecular ions mentioned in the text, protonated molecular ions were also present 22 mass units lower. Signals at 368 and 484 might be β-cleavage fragment ions with the further loss of a methanol moiety.

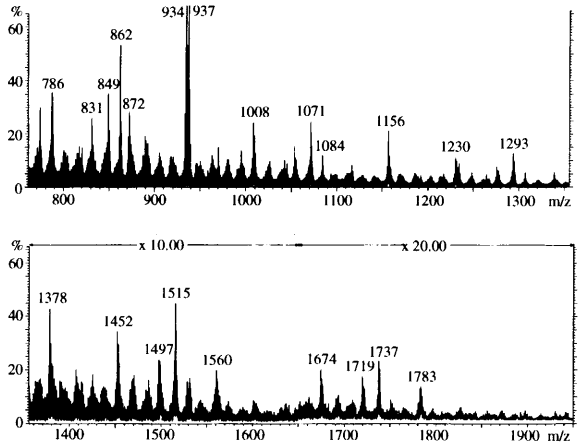


Figure 4 FAB mass spectrum of perdeuteroacetylated acid hydrolysates of AG from *M. bovis* BCG

Only the mass region containing ions of interest in connection with GalNdAc-Ara$_n$ is shown. Other signals of Ara/Gal composition in this mass region were: m/z 862, Gal$_2$Ara$_1^+$; m/z 872, Gal$_1$Ara$_2^+$; m/z 934, Ara$_4^+$; m/z 937, Gal$_3^+$; m/z 1084, Gal$_2$Ara$_2^+$; m/z 1156, Ara$_5^+$; m/z 1378, Ara$_6^+$. The GalNdAc-Ara$_n$ ions were apparently accompanied by dAc adduct ions 45 units higher.

with a UV-absorbing hydrophobic chromophore, ABEE, after N-deuteroacetylation of the free amine group. Excess reagents, together with the salt, were effectively removed by one-step reverse-phase HPLC. Subsequent FAB–MS analysis of the permethyl derivatives resulted in the identification of Gal$_1$, Ara$_2$, Gal$_2$ and GalNHdAc-Ara$_2$, which afforded sodiated molecular ions at m/z 436, 552, 640 and 800 respectively (Figure 3).

Gas chromatography–MS linkage analysis of the alditol acetates derived from this fraction demonstrated the presence of two major peaks identified as terminal Ara*f* and terminal Gal*f*, and two minor peaks of approximately equal abundances identified as 2-linked Ara*f* and terminal GalNAc (results not shown). Because all the reducing-end sugar residues had been converted to ABEE derivatives, Ara-(Ara-ABEE) and Gal-(Gal-ABEE) would yield only terminal Ara and Gal respectively. Thus the only possible source of 2-linked Ara was the GalN-containing component, and its structure must be GalN → 2Ara → (Ara-ABEE). When non-ABEE-tagged fraction B was subjected to similar analysis, alditol acetates of 5-Ara*f*, 3,5-Ara*f*, 5-Gal*f*, 6-Gal*f* and 5,6-Gal*f* were additionally detected, consistent with the published AG structures [20] and indicating that the higher oligomers present in fraction B (Table 1) were a mixture of Ara and Gal fragments derived from the hydrolysis of AG. Interestingly, 2,5-Ara*f*, which was not considered as a constituent residue of AG in the currently accepted model [21], was also detected in relatively high abundance. Although it is possible that the acid hydrolysis conditions might have introduced artifacts, the presence of 2,5-linked Ara suggested that some of the higher oligomers of GalN-Ara$_n$ might be linked as Ara$_n$ → (GalN → 2)5Ara → 5Ara →, i.e. the GalN substituent might occur as a monosaccharide stub branching out at position 2 of the 5-linked arabinan chains of AG.

GalN in other mycobacteria

Limited experiments were done with several other mycobacterial species, aimed at confirming the existence of GalN in the AG and, where it was found, obtaining and examining GalN-containing oligosaccharides. In *M. tuberculosis* Erdman, *M. bovis* BCG and *M. kansasii*, GalN was found in amounts similar to those found in *M. microti*. GalN-containing oligosaccharides were obtained by partial hydrolysis and in these, as in those from *M. microti*, the GalN was not N-acetylated. Detection of GalN-Ara components based on the FAB–MS analysis of chemical derivatives was similarly applied to confirm the presence of such structural entities in hydrolysates of AG from these slow-growing mycobacteria. Briefly, AG was acid- or base-solubilized from the cell wall and further acid-hydrolysed as before, after which the hydrolysates were perdeuteroacetylated for direct FAB–MS analysis without further fractionation. Typically, oligomers of Ara$_n$, Gal$_n$ and Ara$_n$Gal$_n$, either as sodiated molecular ions or oxonium fragment ions, were detected in variable abundances. In addition, two series of ions were also readily detected and could be assigned as deriving from perdeuteroacetylated GalNdAc-Ara$_n$. Thus, as shown in Figure 4, the ions at m/z 849, 1071, 1293, 1515 and 1737 in the FAB mass spectrum of perdeuteroacetylated acid hydrolysates of *M. bovis* BCG AG corresponded to protonated molecular ions of GalNdAc-Ara$_2$, GalNdAc-Ara$_3$, GalNdAc-Ara$_4$, GalNdAc-Ara$_5$ and GalNdAc-Ara$_6$ respectively. Another series of ions at 63 mass units lower was attributable to the oxonium fragment ions of the former series, i.e. GalNdAc-Ara$_n^+$, or elimination of a deuteroacetic acid moiety from the respective molecular ions.

Further confirmation was obtained from FAB–MS analysis of the same samples after additional permethylation, as exemplified by the FAB mass spectrum of the derivatives of *M. tuberculosis* Erdman AG (Figure 5) in which the sodiated molecular ions of permethylated GalNdAcMe-Ara$_2$, GalNdAcMe-Ara$_3$ and GalNdAcMe-Ara$_4$ were shown to be present at m/z 477, 637 and 797 respectively. Another series of ions at m/z 263, 423, 583 and 743 corresponded to the oxonium ions of GalNdAcMe$^+$, GalNdAcMe-Ara$_1^+$, GalNdAcMe-Ara$_2^+$ and GalNdAcMe-Ara$_3^+$ respectively. It was thus clear that the GalN-Ara motif occurred in the AG of all these species.

AG prepared from *M. avium* with either acid or base contained GalN in amounts similar to those found in *M. microti*, which confirms the unpublished work of M. Daffé (personal communication). *M. leprae* AG was shown by amino acid analysis to contain GalN, but the quantities of material available did not permit further examination. None of the rapid-growing mycobacteria examined contained GalN in its AG.

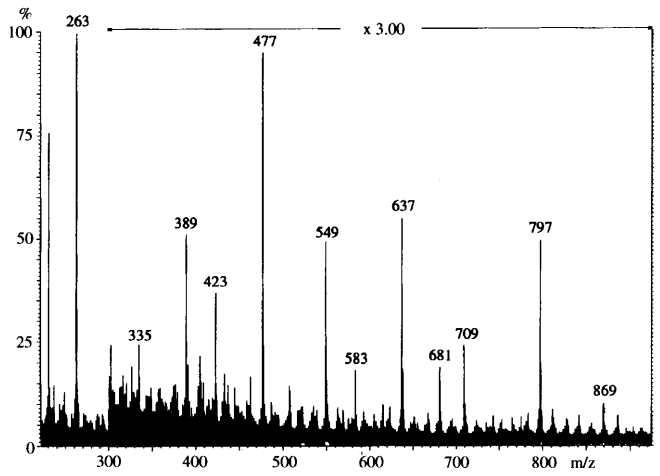


Figure 5 FAB mass spectrum of the permethyl derivatives of per-deuteroacetylated acid hydrolysates of AG from *M. tuberculosis* Erdman

Signals at *m/z* 389, 549, 709 and 869 correspond to sodiated molecular ions of Ara_2, Ara_3, Ara_4 and Ara_5.

DISCUSSION

The amount of GalN in the slow-growing mycobacterial species corresponds to about one hexosamine unit per AG chain. Davidson [5] found equimolar amounts of GalN and phosphate in the walls of *M. microti*, confirming earlier data on *M. lepraemurium* [4] and supporting the suggestion that the GalN might be the part of the linker between the peptidoglycan and the AG, which was already believed to involve a phosphodiester bond (reviewed in [20]). After mild acid hydrolysis the whole of the GalN was found associated with the AG, which was consistent with the action of the acid's being mainly to hydrolyse the phosphodiester link. However, the true composition of the linker has subsequently been determined [21,22]: it involves GlcNAc and rhamnose as well as phosphate, but not GalN.

A preliminary indication that the attachment point might be on the 5-linked arabinan chains of AG was given by methylation analysis of whole cell wall or solubilized AG, when 2,5-linked Ara was detected. This might arise from incomplete cleavage of $GalN^{+}(Me)_3 \rightarrow 2Ara$ bonds under the hydrolysis conditions commonly used in linkage analysis. (Such bonds are relatively resistant to acid hydrolysis because of the strong positive charge on the hexosamine.) Direct permethylation of AG converts the free amine into a trimethylammonium ion, whereas prior protection by N-acetylation prevents the introduction of such a positive charge in subsequent methylation.

The results reported here show that GalN is 2-linked to Ara in the AG as a side chain, i.e. in a position remote from the linker; 2-linked Ara occurs as the penultimate Ara residue in the branched Ara_6 motif at the non-reducing end of AG [23] but apparently nowhere else in the proposed structural model. Previous FAB–MS analyses intended to characterize the endo-arabinanase digestion products of AG ([24], and K. Khoo, D. Chatterjee and M. McNeil, unpublished work) did not detect GalN-Ara motifs in the oligosaccharides released by the enzyme. In a related study (Y. Xin, K.-H. Khoo and M. McNeil, unpublished work), endoarabinanase was used to release preferentially the terminal Ara_6 motifs, leaving a core AG largely devoid of the distinctive t-Araβ1 $\rightarrow$ 2Araα1 $\rightarrow$ signals in its NMR spectrum. With FAB–MS, a putative GalN-Ara motif could be detected in the resistant AG core. Thus the GalN seems to be remote from the non-reducing terminus of AG.

The function of GalN remains unknown. Hexosamines with unacetylated amino groups are uncommon. GlcN occurs with a free amino group in the membrane 'anchors' used by eukaryotic cells to attach certain proteins to membranes [25,26] but there is no report of GalN in this form. The GalN in the mycobacterial AG might bind anionic substances, perhaps helping to stabilize the structure of the envelope. Possible candidate anions are the phosphatidylinositol-based lipomannan and lipoarabinomannan, or the phosphate of the linker itself. Walls of mycobacteria belonging to the *M. tuberculosis* 'complex' and *M. kansasii* commonly contain glutamic acid polymers [27], and although these are largely amidated (i.e. most of the residues are glutamine) [28,29] GalN might bind to the remaining free carboxy groups and so attach the polymer to the wall. Our approach cannot rule out the existence of other saccharide or non-saccharide substituents on hydroxy groups of the GalN itself because the dilute base and dilute acid used in preparing the AG and the GalN-containing oligosaccharides might remove such entities.

Our failure to find GalN in the envelope of the three rapid-growing mycobacterial species examined confirms the unpublished work of M. Daffé (personal communication) and is of interest because it might point to a difference in structure of the envelope between rapid-growing and slow-growing species. At the moment, however, no difference has been reported that might correlate with the absence or presence of a positively charged group in the AG. Because the major mycobacterial pathogens are all slow-growing species, a search for such a difference seems worthwhile.

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