Structural elucidation of the predominant motifs of the major cell wall arabinogalactan antigens from the borderline species *Tsukamurella paurometabolum* and *Mycobacterium fallax*

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Received on December 2, 2004; revised on February 28, 2005; accepted on March 3, 2005

*Tsukamurella paurometabolum* and *Mycobacterium fallax* are members of the suprageneric actinomycte group *Corynebacterineae* that possesses a cell wall skeleton composed of a peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification with C60–C80 mycolic acid residues in mycobacteria and attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached. This polysaccharide is further modified by esterification to peptidoglycan to which an arabinogalactan is covalently attached.

Members of the suprageneric actinomycte group called *Corynebacterineae*, which includes *Mycobacterium, Rhodococcus*, and *Nocardia* genera, possess a chemotype IV cell wall composed of an arabinogalactan covalently attached at one end to peptidoglycan and at the other to mycolic acids (Minnikin and Goodfellow, 1980; Collins et al., 1982; Minnikin, 1982; Brennan and Nikaio, 1995; Daffe and Draper, 1998). The arabinogalactan component has been implicated in numerous biological activities associated with human and experimental mycobacteria, such as the high titer IgG antibodies in tuberculosis and leprosy sera (Misaki et al., 1974; Miller et al., 1984) and the T-cell-mediated immunity (Kleinhenz et al., 1981). Arabinogalactan, unlike most polysaccharides, is devoid of a repeating unit (Daffe et al., 1990, 1993). Its structural features were first established for *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, through the characterization of oligoglycolyl alditol fragments derived from per-O-methylation of the cell walls, partial hydrolysis, reduction, pentadeterioethylation (Daffe et al., 1990). The fractionation of the resulting oligoglycolyl alditols by high performance liquid chromatography and analysis of anomer configurations by NMR and pattern fragmentations by gas chromatography–mass spectrometry (GC-MS) enabled the recognition of several structural motifs, which in turn, allowed the elaboration of a tentative structural model for the complex molecule (Daffe et al., 1990). The polysaccharide from *M. tuberculosis* appeared to be composed of two distinct entities: an homogalactan portion, composed of alternating 5-linked β-galactosyl furanosyl (β-Gal f) and 6-linked β-galactosyl furanosyl (β-Araf) disaccharide branched on both position 3 and position 5 of an α-Araf unit, and (4) a 5-linked-α-Araf unit branched on both position 3 and position 5 of an α-Araf residue. The polysaccharide from *T. paurometabolum* possesses additional structural domains composed of a terminal (r) Araf directly linked to either a 5-linked-α-Araf or to both position 3 and position 5 of a 3,5-linked-α-Araf unit. Both the remarkable similarity of...
to the conclusion that all the mycobacterial products, whatever the pathogenicity and the growth conditions of the strains examined, for example, in vivo versus in vitro, optimal temperature of growth, were similar to those of *M. tuberculosis* (Daffe et al., 1990, 1993). In contrast, arabinogalactans from other genera contained genera- or species-specific structural motifs (Daffe et al., 1993). For instance, the backbone of the galactan portion of the cell wall arabinogalactans from both *Nocardia asteriodes* and *Nocardia brasiliensis* consisted of linear 5-linked β-Galf units and was devoid of 6-linked Galf residues. This latter type of glycosyl unit was present in *Rhodococcus equi* but absent from the arabinogalactan of *Rhodococcus rhodochrous* which contains, in addition to the 5-linked Galf, 2-linked Galf but not the 3-linked Galf that typifies *R. equi* (Daffe et al., 1993).

Arabinogalactans of members of the *Corynebacterineae* suborder are esterified by these α-branched, β-hydroxylated long-chain (up to C90) fatty acids, called mycolic acids, whose structures are widely used as a source of valuable information for the determination of genera within the *Corynebacterineae* group (Minnikin and Goodfellow, 1980; Collins et al., 1982; Minnikin, 1982; Brennan and Nikaido, 1995; Barry et al., 1998; Daffe and Draper, 1998). Within this group, mycobacteria elaborate mycolates possessing the highest number of carbon atoms (C70–C90) and produce several types of mycolic acids, which differ one from another by the occurrence of chemical groups that are not found in other bacterial genera, such as cis and trans cyclopropane rings, methyl branches, keto-, methoxy-, hydroxy-, and epoxy-group, and carboxy functions. An additional distinctive feature of mycobacteria resides in the fact that their most common type of mycolates, called α-mycolates, contains only two double bonds or two cyclopropane rings, which may have adjacent methyl branches (Minnikin, 1982; Brennan and Nikaido, 1995; Barry et al., 1998; Daffe and Draper, 1998). In contrast, tri- and tetra-enoic C40–C60 mycolic acids have been described in the well-characterized *Rhodococcus* and *Nocardia* genera of the *Corynebacterineae* suborder (Minnikin and Goodfellow, 1976; Barry et al., 1998). Some previously misidentified strains of *Rhodococcus* and *Corynebacterium*, as well as members of the proposed *Gordona* and *aurantiaca* taxons, have been recently grouped in the new genus, *Tsukamurella*, based on their 16S RNA sequences (Collins et al., 1988). The latter bacteria were found to produce only α-type mycolates, which contain one to six double bonds, with chain lengths similar to those of mycobacteria (C60–C80) (Collins et al., 1988). Interestingly, a single mycobacterial species, *Mycobacterium fallax*, has been described to elaborate α-type mycolates, C70–C90, possessing up to five double bonds (Levy-Frebault et al., 1983; Rafidinarivo et al., 1985). Accordingly, *M. fallax* and *Tsukamurella* are considered as borderline species in at least three bacterial genera, namely *Mycobacterium*, *Rhodococcus*, and *Nocardia* (Papa et al., 1986). This study addresses the question of the species- or genus-specificity of the structure of the cell wall arabinogalactans of *Corynebacterineae* through the determination of the structural motifs that composed the cell wall polysaccharides of *Tsukamurella paurometabolum* and *M. fallax*.

### Results

#### Purification, glycosyl composition, and linkage analysis

Intact cell walls of *M. fallax* and *Tsukamurella* were acid hydrolyzed followed by trimethylsilylation and GC analysis.
Table 1. Glycosyl-linkage analysis of arabinogalactans from *Mycobacterium fallax* and *Tsukamurella paurometabolum*

<table>
<thead>
<tr>
<th>Glycosyl residue</th>
<th>Mycobacterium tuberculosis</th>
<th>Mycobacterium fallax</th>
<th>Tsukamurella paurometabolum</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-Araf</td>
<td>11.0</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>2-Araf</td>
<td>11.0</td>
<td>11.0</td>
<td>8.0</td>
</tr>
<tr>
<td>5-Araf</td>
<td>37.0</td>
<td>36.0</td>
<td>30.0</td>
</tr>
<tr>
<td>3,5-Araf</td>
<td>11.0</td>
<td>15.0</td>
<td>17.0</td>
</tr>
<tr>
<td>t-Gal</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5-Gal</td>
<td>17.0</td>
<td>18.0</td>
<td>25.0</td>
</tr>
<tr>
<td>6-Gal</td>
<td>9.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>5,6-Gal</td>
<td>2.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*The glycosyl-linkage composition of Mycobacterium tuberculosis is from (Daffe et al., 1990) and is given for comparison.*

Seroreactivities of the arabinogalactans from *M. fallax* and *T. paurometabolum* with the anti-arabinogalactan antibodies from *Mycobacterium bovis*

The antigenicity of mycobacterial cell walls is well established and arabinogalactan is known to be an elicite a strong humoral response of mammalian host cells (Misaki et al., 1974). It has been shown that arabinosyl residues are responsible for the antigenicity of arabinogalactans from mycobacteria and related species and that most of the serological activity resides in a fraction containing 2-Araf residues (Kotani et al., 1971; Misaki et al., 1974), that is, part or all structural motif A. Accordingly, it was possible to evaluate the structural similarities between various arabinogalactans by measuring their seroreactivities. For this purpose, polyclonal antibodies were raised against the arabinogalactan–peptidoglycan complex from *M. bovis* and incubated with competitors consisting of polysaccharides purified from either the cell walls of *M. bovis*, *M. fallax*, or *T. paurometabolum*. As expected, the arabinogalactan from *M. bovis* was capable to completely inhibit the reaction between the antibodies directed against the arabinogalactan–peptidoglycan complex from the same species (Figure 2). Although a similar pattern of inhibition was observed with the polysaccharide from *M. fallax* (data not shown), the arabinogalactan from *T. paurometabolum* strongly inhibited the seroreaction (Figure 2). Thus, these data showed that the antigenic domains of the polysaccharide of *M. fallax*, that is, the arabinan segments, are serologically distinguishable from those of *M. bovis* bacille calmette guérin (BCG) and suggested closely related structures for the arabinan domains of *M. fallax* and *T. paurometabolum*. However, because the inhibition of the seroreaction by the arabinogalactan derived from *T. paurometabolum* was not completed, quantitative and/or qualitative differences might exist between the arabinan segments of the latter species and those from mycobacteria.

Analysis of the $^{13}$C NMR spectra of the arabinogalactans from *M. fallax* and *T. paurometabolum*

To decipher the structural features of the arabinogalactans from the two strains, we analyzed the purified polysaccharides by NMR. As expected from the glycosyl compositions, the $^1$H NMR spectra of the arabinogalactans from *M. fallax* and *T. paurometabolum* were similar to one another (data not shown) and to those of mycobacteria and rhodococci but different from those of nocardiae (Daffe et al., 1990, 1993). Anomeric proton signals of the arabinogalactans from *M. fallax* and *T. paurometabolum* were seen at $\delta$ 5.0–5.3.
consistent with the presence of α- and/or β-furanosyl residues and/or α-pyranosyl units (Daffe et al., 1993). Resonances of the other sugar protons were grouped at δ 4.3–3.6 (data not shown). Because 13C NMR has proved to be an excellent investigative tool, both as an aid in structural determination and in fingerprinting for taxonomic purposes (Daffe et al., 1990, 1993), the purified arabinogalactans were comparatively analyzed by this technique (Figure 3).

The 13C NMR spectrum of the arabinogalactan from M. fallax (Figure 3A) was almost superimposable to that of other mycobacteria (Daffe et al., 1990, 1993) whereas that from T. paurometabolum presents many similarities with that of the product of M. tuberculosis (Daffe et al., 1990). Based on the previous assignments deduced for the spectra of M. tuberculosis (Daffe et al., 1990) and other Corynebacterineae (Daffe et al., 1993), resonances attributable to the C-1s of t-β-Araf and 2-linked-α-Araf of the nonreducing end of the arabinogalactan molecules were seen at 101–102 and 106–108 ppm, respectively. The resonances of the C-2s of the 2-Araf were identified at δ 88.2 and 87.9 whereas that of the C-5 of t-β-Araf was seen at 64.1 ppm (Daffe et al., 1990, 1993). The occurrence in the two spectra of arabinogalactans of two pairs of signals assignable to the C-1 resonances of two t-β-Araf and two 2-linked-α-Araf in slightly different chemical environments, that is, one t-β-Araf or 2-linked-α-Araf, is attached to C-3 and the other to C-5 of the 3,5-linked-α-Araf residue (Daffe et al., 1990, 1993), supported the existence of structural motifs A and B (Figure 1) in the polysaccharides from M. fallax and T. paurometabolum. The intensities of the pairs of signals corresponding to C-1 resonances of t-β-Araf and 2-linked-α-Araf and C-2 resonances of 2-linked-α-Araf are approximately equal in the spectrum of the arabinogalactan from M. fallax but not in that of T. paurometabolum, suggesting that both types of Ara residues are engaged in other structural motifs in the arabinogalactan of the latter species. The furanoid nature of remaining Ara and Gal residues was also obvious from the chemical shift values of the C-1 resonances (δ 108–110). The resonances of primary alcohol carbons of unsubstituted α-Araf and β-Gal were observed at δ 61–62 whereas those of 5- and 3,5-Araf and 6- and 5,6-Gal were seen at δ 70–72. These assignments were based on the glycosyl linkage composition (Table I) and 13C NMR spectroscopy in the distortionless enhancement by polarization transfer (DEPT) mode (Figure 3), in agreement with the literature data (Daffe et al., 1990, 1993). The assignment of the remaining 13C NMR resonances can be found in Daffe et al. (1993).

Structures of per-O-alkylated oligoglycosyl alditol fragments from the arabinogalactans

Aliquots of the per-O-methylated cell walls were partially hydrolyzed with acid, reduced with NaBD₄, pentadeuterioethylated and the resulting per-O-alkylated oligoglycosyl alditol fragments were analyzed by GC-MS; their structures were deduced from the analysis of mass spectra, based on those of the well-characterized oligoglycosyl alditol fragments obtained from the arabinogalactan of M. tuberculosis (Daffe et al., 1990). The method used for the structural elucidation of per-O-alkylated oligoglycosyl alditol fragments has been previously described (Daffe et al., 1990, 1993). The confirmation of the furanoid nature of Ara units came from the pentadeuterioethylation of the hydroxyl groups on C-1 and C-4. The location of a pentadeuterioethyl group on an hydroxyl group at positions other than at C-1 and C-5 of the arabinitol demonstrated the substitution of this residue at this position in the native arabinogalactan. An illustration of the application of these principles and the corresponding interpretation of the mass spectra are shown in Figure 4 for two per-O-alkylated oligoglycosyl alditol fragments arose from the series of degradation/modifications of the arabinogalactans of M. fallax and T. paurometabolum (fragments 5 and 7, Table II). The two fragments corresponded to a 2-Araf and a 5-Araf residues linked at position 3 of a 3,5-substituted-Araf in the polysaccharides. Upon the series of degradation/modifications, fragment 5 will bear methoxyl groups at unsubstituted positions C-3 and C-5 of the 2-Araf residue and position C-2 of the alditol. The pentadeuterioethyl groups will be located on hydroxyl groups that were occupied in the arabinogalactan and liberated by the series of degradation/modifications. Thus, they will be located on position C-2 of the 2-Araf residue and positions C-1 and C-4, which were engaged in the furanoid, and position C-5 of the alditol. The same reasoning is valid for fragment 7, except that the methoxyl groups will be

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**Fig. 3.** 13C NMR spectra of the base-solubilized cell wall arabinogalactans from Mycobacterium fallax (A) and Tsukamurella paurometabolum (B). The spectra were obtained with a distortionless enhancement by polarization transfer (DEPT) pulse sequence and recorded in 2H₂O (40 mg/mL) at 125.7 MHz. *denotes contaminant peak.
Arabinogalactans of *M. fallax* and *T. paurometabolum* located at positions C-2 and C-3 of the 2-Araf residue (and position C-2 of the alditol) whereas the pentadeuteroethyl groups will be located on C-5 of the Araf residue (and positions C-1, C-4, and position C-5 of the alditol). The two mass spectra of the per-O-alkylated oligoglycosyl alditols were very similar to one another and shared most peaks; they differed, however, by the \( m/z \) value of their prominent peak, at 120 and 101 \( m/z \) for fragments 5 and 7, respectively. These peaks arose from a rearrangement process and contain C-1, C-2, and C-3 (Kochetkov and Chizhov, 1966). The \( m/z \) value of the arabinosyl unit in both fragments (at \( m/z \) 194) demonstrated that they contained two methoxyl and one pentadeuteroethoxyl groups. The loss of methanol (32 mass units) from both arabinosyl residues showed that a methoxyl group was located on position C-3 (Kochetkov and Chizhov, 1966). Finally, the occurrence of a prominent peak at \( m/z \) 120 proved that C-2 in fragment 5 bore the pentadeuteroethoxyl group (Figure 4). This latter group was necessarily located on C-5 in fragment 7 because the prominent peak was observed at \( m/z \) 101, corresponding to a fragment that contained C-1, C-2, and C-3 and two methoxyl groups. That the arabinosyl unit in both fragments 5 and 7 was linked to C-3 of the per-O-alkylated alditol was deduced from the absence of the peak at \( m/z \) 287 from their mass spectra but present in those of fragments 6 and 8 (data not shown). This peak arose from the fragmentation between the C-3 and C-4 of the per-O-alkylated alditol and its \( m/z \) mass value corresponds to a fragment that contained the per-O-alkylated arabinosyl residue, the C5 of the alditol to which it is linked, and its C-4 which bears a pentadeuteroethoxyl group. Such a fragmentation can be seen only in 5-linked, but not in 3-linked, per-O-alkylated alditol. As only 3- and 5-substituted branched arabinosyl units and alditols occur in the arabinogalactans analyzed (Table I), fragments 5 and 7 have the structures depicted in Table II.

The examination of the structures established for the oligoglycosyl alditol fragments showed that, like mycobacteria and related genera (Daffe *et al.*, 1990, 1993), the arabinogalactans from the two species examined consist of arabinan and galactan regions because most of the fragments contain only Ara and Gal (rather than a mixture of both).
It was possible also to recognize several of the oligosaccharide families previously found in the degradation/modification products of arabinogalactans from *Corynebacterineae* that have led to the definition of structural motifs. For instance, the occurrence of motif A, composed of α-Araf substituted at both position 3 and position 5 with the t disaccharide...
β-Ara-f-(1→2)-α-Araf (Figure 1), in the arabinogalactans from *T. paurometabolum* could be deduced from the identification of oligoglycosyl alditols 1, 3, 5, 6, 10, 11. For *M. fallax*, the identification of compounds 10 and 11 confirmed the occurrence of motif A, the occurrence of which was already demonstrated by the analysis of the $^{13}$C NMR spectrum of its arabinogalactan (see section “Analysis of the $^{13}$CNMR spectra of the arabinogalactans from *M. fallax* and *T. paurometabolum*). Indeed, two pairs of signals, seen at 101–102 and 106–107 ppm, were assignable to the C-1 resonances of two t-β-Araf and two 2-linked-α-Araf, respectively, in slightly different chemical environments (Daffe et al., 1990, 1993). Accordingly, the reason for the absence of some of the characteristic fragments of the structural motif A, and more generally of a given motif, in *M. fallax* was not investigated further, due to the fact that the $^{13}$C NMR spectrum of the arabinogalactan of the species was superimposable to those of mycobacterial species previously examined in detail (Daffe et al., 1990, 1993). Similarly, the presence of oligoglycosyl alditols 7 and 8 in both *M. fallax* and *T. paurometabolum* and of compound 20 in the latter species established the occurrence in the arabinogalactans examined of structural motif B, a 3,5-linked-α-Araf substituted with 5-linked-α-Araf at both branched points (Figure 1). The identification of compounds 13, 14, and 15 in the mixtures of oligoglycosyl alditols showed that the 5-Ara units that substitute the 3,5-Ara units within motif B are linked to adjacent 5-Ara residues. Likewise, as expected from the large amounts of 5-Ara residues in the arabinogalactans (Table I), the existence of compounds 4 and 12 implied the occurrence of motif C, a linear 5-linked α-Araf (Figure 1). In the mycobacterial arabinogalactans examined so far the homogalactan domains consist of alternating 5-linked β-Gal/ and 6-linked β-Gal/ units, corresponding to structural motif D. The occurrence of such a motif in the polysaccharide of *M. fallax* was supported by the identification of compounds 28, 29, and 30 (Table II). That motif D occurs also in *T. paurometabolum* was indicated by the characterization of compound 28 as the only per-O-alkylated Gal galactitol in the mixture of degradation/modification products of the arabinogalactan of the species. Under similar analytical conditions, this compound is by far the major Gal galactitol derivative found in mycobacteria (Daffe et al., 1993). Nevertheless, because the ratio 5- to 6-linked Gal/ is especially high in the arabinogalactan of *T. paurometabolum*, compared with that observed in the polysaccharides from both *M. tuberculosis* and *M. fallax* (Table I), the existence of a motif consisting of linear 5-linked Gal/ in the galactan domain of *T. paurometabolum* was suspected and investigated. However, a careful examination of the mass spectra of the oligoglycosyl alditols, notably by selected ion monitoring, did not allow the identification of compounds that would support the occurrence of any additional structural motif in the galactan backbone of *T. paurometabolum*. Unfortunately, analysis of the $^{13}$C NMR spectrum of the arabinogalactan revealed no clue that might shed some light on this aspect. It was thus concluded that the arabinogalactan from *M. fallax* contains of a homogalactan segment of alternating 5- and 6-linked Gal/ and that the galactan of *T. paurometabolum* is similar, at least in part, to that of *M. fallax* and other mycobacterial species examined so far (Daffe et al., 1990, 1993).

Oligoglycosyl alditols absent from the mycobacterial arabinogalactan products were found in those of *T. paurometabolum* (Table II), consistent with the analysis of the $^{13}$C NMR spectra (Figure 3B). The presence of fragments i and vi in the arabinogalactan degradation/modification products of *T. paurometabolum* established the occurrence of motif A’ that contains t-Araf residues directly linked to both position 3 and position 5 of 3,5-Ara units, as already observed in *R. equi* (Daffe et al., 1993). Presumably these t-Araf residues represent what in motif A are 2-α-Araf residues and have an α configuration (at δ 108–109). However, the intensities of signals at δ 101–102 and the multiplicity of those at δ 106–107 and δ 88–89 in the $^{13}$C NMR spectrum of *T. paurometabolum* (Figure 3B) indicated that, at times, motif A’ may be modified by the addition of a single t-β-Araf unit. In addition, compound ii was identified in the degradation/modification products of the arabinogalactan from *T. paurometabolum* (Table II), indicating that t-Araf may also be linked to 5-Araf in a novel motif A’”. We thus concluded that three non-reducing termini structural motifs, namely A, A’, and A”, exist in the arabinan domains of *T. paurometabolum*. Unexpectedly, compound ii was also observed in the degradation/modification products of the arabinogalactan from *M. fallax* (Table II) but probably represents a minor product because the intensities of the pair of signal resonances at δ 101–102 (Figure 3B) in the $^{13}$C NMR spectrum of the arabinogalactan from *M. fallax* were comparable with those found in mycobacterial polysaccharides that are devoid of motif A”.

**Discussion**

Mycobacterium fallax and *T. paurometabolum* are two members of the *Corynebacterineae* suborder which is composed of bacteria possessing a chemotype IV peptidoglycan, an arabinogalactan and mycopic acids. However, the two bacterial species appeared to be borderline species in that they produce the long-chain α-mycolate type of mycobacteria but these molecules may contain more than two double bonds, a feature that typifies *Rhodococcus* and *Gordonia*. Consistent with this observation is the phylogenetic tree based upon nucleotides of 16S rRNA from *Corynebacterineae* which showed that the type strain of *Tsukamurella* was located between *Mycobacterium* and *Gordonia* genera (Embley and Stackebrandt, 1994). Indeed, previous studies have shown that the structural features of the cell wall arabinogalactans from some bacterial genera that composed the *Corynebacterineae* suborder are species- or genus-specific (Daffe et al., 1990, 1993). To challenge this latter concept, we examined the major structural domains of the arabinogalactans from the borderline species *M. fallax* and *T. paurometabolum*.

The application of acid-catalyzed partial treatment to the per-O-alkylated cell wall polysaccharides and analysis of the resulting oligosaccharides allowed us to define the major structural features of the arabinogalactans from *M. fallax* and *T. paurometabolum*. Both species elaborate polysaccharides that contain the different structural motifs
previously characterized in the arabinogalactans of various mycobacteria. These consist of (1) a nonreducing pentaarabinosyl composed of a β-Araf-(1→2)-α-Araf/ disaccharide branched on both position 3 and position 5 of an Araf unit (motif A'), (2) a 5-linked-Araf unit branched on both position 3 and position 5 of an Araf residue (motif B), (3) a linear 5-linked Araf (motif C), and (4) an alternating 5-linked β-galactofuranosyl and 6-linked β-galactofuranosyl residues (motif D). No additional structural motif was found in the arabinogalactan of *M. fallax*, consistent with the seroreactivity of the arabinogalactan with antibodies directed against the cell walls of *M. bovis*. Thus, despite its distinctive polynoic mycolate content, the cell wall arabinogalactan from *M. bovis* has a structure very similar to that of other examined mycobacteria (Daffe et al., 1990, 1993). The elucidation of the major motifs of the arabinogalactan from *T. paurometabolum* demonstrated the existence of two nonreducing termini in the arabinan segment not found in mycobacterial polysaccharides; these consist of t-Araf residues directly linked to both position 3 and position 5 of 3,5-Araf units (motif A') and t-Araf/linked to a linear oligoarabinosyl composed of 5-Araf residues (motif A''). Interestingly, structural motif A' has been found in the arabinogalactan from some species of *Rhodococcus* (Daffe et al., 1993), a genus that elaborates also polyenoic mycolic acids (Collins et al., 1988; Barry et al., 1998) and is phylogenetically close to *Tsukamurella*. It has to be noted, however, that the distinctive structural motifs of the arabinogalactan of *T. paurometabolum* are not found in the structurally related lipoarabinomannan of the species whose arabinan segment consists only of a linear 5-linked Araf (motif C) (Gibson et al., 2004).

Previous structural studies on the arabinogalactans from several mycobacterial species have shown that the structures of the polysaccharides from both in vivo- and in vitro-grown strains, whatever their pathogenicity and the growth temperatures, were similar to that of *M. tuberculosis* (Daffe et al., 1990, 1993). The arabinogalactans from representative strains of *Rhodococcus* and *Nocardia* genera have been shown to share structural motifs with those of mycobacteria and to produce genus- or species-specific motifs (Daffe et al., 1993). Therefore, this study reinforces the concept that members of the suprageneric group *Corynebacterineae* produce remarkably conserved cell wall arabinogalactans. Interestingly, NMR data obtained on the native polysaccharides confirmed the structural similarities observed between the different arabinogalactans and, more importantly, showed some differences that typify each of the polysaccharides according to the origins. Accordingly, the use of 13C NMR spectra of arabinogalactans as a fingerprint would help in the identification or confirmation of the validity of borderline actinomycete species.

Although all the structural motifs found in mycobacterial arabinogalactans were also present in the polysaccharide from *T. paurometabolum*, the arabinogalactan antigen from this species was not capable to completely inhibit the seroreaction of the antibodies directed against the cell walls of *M. bovis*. Indeed, this observation could be explained by the occurrence of additional antigenic structural motifs in the *M. bovis* cell wall polysaccharide that would be absent from the arabinogalactan of *T. paurometabolum*. However, this hypothesis is not supported by the detailed structural analyses of the polysaccharides isolated from different mycobacterial species (Daffe et al., 1990, 1993), and consequently unlikely. Based on the assumption that the serological activity of the mycobacterial arabinogalactan resides largely in a fraction containing 2-linked arabinosyl residues (Kotani et al., 1971; Misaki et al., 1974), an alternative explanation of the uncomplete inhibition of the seroreaction by the arabinogalactan of *T. paurometabolum* would be that motif A is a minor component of the arabinan termini. As a consequence, more arabinogalactan antigen from *T. paurometabolum* would be needed to achieve the inhibition than the case of mycobacteria. However, the examination of the inhibition curve questions this hypothesis because the amount of antigen did not correlated with the percentage of inhibition. This phenomenon may then be due to conformational reasons, for example, the presence of motifs A' and A'', which are virtually devoid of antigenicity, may hamper the seroreaction between the antigenic termini of arabinogalactan and antibodies.

**Materials and methods**

**Bacterial strains and culture conditions**

The type strains of *M. fallax* [Collection Institut Pasteur (CIP) 8139, American Type Culture Collection (ATCC) 35219, or Collection Institut Pasteur Tuberculose (CIPT) 1390005] and *T. paurometabolum* [ATCC 8368, Japan Collection of Microorganisms (JCM) 7663, or Deutsche Sammlung von Mikroorganismen (DSM) 20162] were grown on Nutrient Broth (Difco, Detroit, MI) medium for 2 weeks at their optimal temperature of 30°C and 37°C, respectively. Cell suspensions were autoclaved and cells recovered by centrifugation (20 min at 5000 rpm), washed, and frozen at −20°C.

**Cell wall production**

Cell walls were prepared as previously described (Daffe et al., 1990, 1993). Briefly, wet cells were resuspended in phosphate buffer (50 mM, pH 7.2) at the concentration of 5 g in 20 mL and broken in a cell disrupter (2.7 Kbar) or by sonication. After 3 cell disrupter cycles, intact cells were removed by centrifugation (3000 g) and the supernatant containing 5.2 M L of 5.2 M C, respectively. Cell wall pellets were treated with aqueous 2% SDS at 95°C for 1 h and pelleted again at 27,000 g. Cell walls were then washed twice with 80% (v/v) acetic acid in water, and then water, to remove sodium dodecyl sulfate and lyophilized. The glycosyl composition of purified cell walls was determined by hydrolyzing an aliquot with 2 M CF3COOH for 2 h at 110°C, followed by trimethylsilylation (Sweeley et al., 1963) and GC analysis of the resulting products.

**Methylation of the cell wall arabinogalactans**

Cell walls were O-methylated by a slight modification of the Hakomori procedure (York et al., 1986). Cell walls (100 mg) were suspended in 3 mL of dimethylsulfoxide (Pierce Chemical Company, Rockford, IL), then 300 µL of 5.2 M dimethylsulfonyl carbanion added, and the mixture stirred...
for 1 h. An equimolar amount of CH$_3$I (120 µL, Aldrich Chemical Co., Milwaukee, WI) was slowly added and the mixture was stirred for 1 h. The addition of the base and CH$_3$I was repeated and an excess (2 mL) of CH$_3$I was added at the end of the third cycle. After 1 h of stirring, the reaction mixture was partially evaporated with N$_2$, an equal volume of H$_2$O added and the mixture dialyzed and the retentate was lyophilized. Aliquot fractions of per-O-methylated cell walls were hydrolyzed with 2 M CF$_3$COOH for 2 h at 110°C, reduced with NaBD$_4$, and acetylated. The resulting per-O-methylated alditol acetates were analyzed by GC-MS as previously described (Daffe et al., 1990, 1993).

Production and analysis of per-O-alkylated oligoglycosyl alditols

To obtain partially O-methylated oligoglycosyl alditol fragments for the linkage analysis, we subjected the per-O-methylated cell walls to partial acid hydrolysis with 2 M CF$_3$COOH for 1 h at 75°C, followed by reduction with NaBD$_4$, and pentadeterioethylation by using the Hako-mori alkylation procedure with C$_2$D$_3$I (Aldrich Chemical Co.). The extend of hydrolysis of the glycosidic linkage of each glycosyl residue was determined by NaBD$_4$ reduction, complete hydrolysis, NaBH$_4$ reduction, acetylation, and GC-MS analysis. Based on these results, the remaining per-O-alkylated cell walls were hydrolyzed for an additional 1 h at 75°C with 2 M CF$_3$COOH, the products reduced with NaBD$_4$ and pentadeterioethylated. The resulting products were the source of the per-O-alkylated oligoglycosyl alditols.

GC-MS of the per-O-alkylated oligoglycosyl alditol fragments were performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5970 mass selective detector. The temperature of the injector was 290°C and the transfer line was 280°C. The column was a 12 m HP-1 (Hewlett-Packard, Avondale, PA). The oven was programmed to hold at 50°C for 1 min, followed by a 30°C/min rise to 200°C, and 8°C/min rise to 320°C and a 8-min hold. The mass spectrometer was set to scan from 50 to 800 atom mass units.

Purification of arabinogalactans and NMR analysis

For NMR analyses, purified cell walls were treated with 2 M NaOH (16 h, 80°C) to cleave the phosphodiester link between arabinogalactan and peptidoglycan (Daffe et al., 1990, 1993). The supernatant, which contained the soluble arabinogalactan, was obtained by centrifugation (27,000 g for 30 min), neutralized with acetic acid, and dialyzed to remove salts. A precipitate formed during the treatment was removed by centrifugation. The supernatant was treated with ethanol (80% in water) and kept at −20°C overnight to precipitate the polysaccharide that was recovered by centrifugation and lyophilized. The glycosyl composition of the polysaccharides was determined by hydrolyzing an aliquot with 2 M CF$_3$COOH for 2 h at 110°C, followed by trimethylsilylation (Sweeley et al., 1963) and GC analysis of the resulting products.

For $^1$H and $^{13}$C NMR analysis, arabinogalactans were solubilized in $^2$H$_2$O (20 mg/0.5 mL) and analyzed at 500 and 125.7 MHz, respectively, on a Bruker AMX 500. $^1$H and $^{13}$C decoupled $^{13}$C spectra were obtained with a DEPT pulse sequence.

Immunological procedures

Rabbit antiserum was obtained by intradermal injection of 1 mL of a water-in-oil emulsion by mixing 0.5 mL PBS containing 5 mL of purified cell walls of M. bovis (Daffe et al., 1993) and 0.5 mL Freund’s incomplete adjuvant (Lopez-Marin et al., 1994). The emulsion was injected into 3-month-old New Zealand white rabbits. Three weeks later, the animals received a booster dose of 3 mg cell wall in water-in-oil emulsion, as previously mentioned, and were bled at 2-week intervals.

The inhibition of the reaction between solid-phase cell walls of M. bovis and specific rabbit (anti-cell walls of M. bovis) serum by arabinogalactan from either M. fallax or T. paurometabolum was performed as follows. Cell walls of M. bovis were suspended in 1 mg/mL of a phosphate buffer. Samples of the suspension (50 µL) were applied to the wells of a polystyrene microtitre plate (Nunc-Immuno plate I) and allowed to evaporate at 37°C overnight. Wells were saturated by adding 200 µL of 30% (w/v) powdered skimmed milk in phosphate buffer containing 0.1% Tween and the plates were incubated for 2 h at 37°C. After 5 washings of the wells with 1% (w/v) powdered skimmed milk in phosphate buffer, the inhibitory solutions were added to the wells. The latter solutions consisted of 200 µL of arabinogalactan antigen from either M. fallax or T. paurometabolum in 500, 250, 125, or 100 µg/mL of phosphate buffer and 20 µL of specific rabbit (anti-cell walls of M. bovis) serum diluted 1:5000 in phosphate buffer. The reaction was incubated for 2 h at 37°C before rinsing. The colour reaction used anti-rabbit IgG (Sigma, St. Louis, MO) and p-nitrophenyl phosphate substrate (Sigma) as previously described (Munoz et al., 1998).

Acknowledgement

M. Daffe is indebted to Dr. Mike McNeil (Colorado State University, Fort Collins) for initiating him in the early 90s into the fascinating field on structural elucidation of complex carbohydrates through a degradation/modification strategy.

Abbreviations

Ara, arabinosyl; DEPT, distortionless enhancement by polarization transfer; f, furanosyl; Gal, galactosyl; GC, gas chromatography; GC-MS, gas chromatography–mass spectrometry; t, terminal.

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