Extracellular and surface-exposed polysaccharides of non-tuberculous mycobacteria

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We studied the outermost constituents of the cell envelopes, which are involved in the interaction between the bacilli and the host cells, of five pathogenic and non-pathogenic mycobacterial species for comparison with those we have previously characterized from M. tuberculosis. The extracellular materials (ECMs) were isolated by ethanol precipitation and compared to the surface-exposed materials (SXMs) extracted by mechanical means. The materials from both sources were composed almost exclusively of polysaccharides and proteins. Two groups of mycobacteria were clearly distinguishable. The first group comprised the pathogenic species M. kansasii which produced large amounts of ECM, the glycosyl composition of which was similar to that of the SXM. The second group comprised M. avium and the non-pathogenic strains of M. gastri, M. phlei and M. smegmatis which produced small amounts of ECM. This latter group could be subdivided into those which produced carbohydrate-rich ECM (M. avium and M. gastri) and those forming protein-rich ECM (M. phlei and M. smegmatis), a classification that correlated with the difference in the growth rate of the two subgroups. The glycosyl composition of the ECM of a given species was qualitatively similar to that of the SXM, except for M. avium and M. phlei whose SXM were devoid of arabinose. In addition to glucose, mannose and arabinose, xylose was detected in the hydrolysis products of the ECM and SXM of M. smegmatis, the SXM of M. phlei and the ECM of some batches of M. avium. The polysaccharide constituents of the ECM and SXM of the different mycobacteria were purified by anion-exchange and gel-filtration chromatography; all were found to be neutral compounds devoid of acyl substituents. The extracellular polysaccharides consisted of high-molecular-mass glycogen-like glucans, arabinomannans and mannans, structurally similar to the corresponding substances previously characterized from the capsule of M. tuberculosis. The same types of polysaccharides were characterized from the SXM of all the strains, except M. avium and M. phlei which were devoid of arabinomannans. This study questions the unique and universal representation of the mycobacterial cell envelope and the existence of the so-called acidic polysaccharide-rich outer layer.

Keywords: arabinomannan, cell envelope, glucan, mycobacteria, polysaccharides

INTRODUCTION

Among the infectious diseases, those resulting from infections with Mycobacterium species are important sources of morbidity and mortality throughout the world today. Almost 2 billion people have been infected with Mycobacterium tuberculosis, the causative agent of tuberculosis, of whom 8 million develop active disease and

Abbreviations: AM, arabinomannan; ECM, extracellular material; SXM, surface-exposed material; SmT, smooth transparent; SmD, smooth domed-opaque.
approximately 3 million people die each year from this disease (Bloom & Murray, 1992; Kochi, 1991). Mycobacterial infections have also re-emerged as an important public health problem worldwide, and this resurgence has been accompanied by an increased incidence of tuberculosis resistant to the standard antituberculosis drugs. Furthermore, and as a result of the human immunodeficiency virus epidemic, it has been learned that HIV infection accelerates the natural history of new M. tuberculosis infection (Chaisson, 1993) and that HIV-infected individuals are more susceptible to reactivation of a previously dormant focus of the tubercle bacillus (Chapman & Henderson, 1994), resulting in a high incidence of tuberculosis. In addition, disseminated mycobacterial infections, notably those caused by members of the so-called M. avium–intracellulare complex, occur in a large proportion of HIV-infected persons.

Mycobacteria are appreciably more resistant than other bacteria to deleterious agents, such as acids, alkalis and germicides (Youmans, 1979), a property commonly attributed to the inner insoluble matrix of the mycobacterial cell wall which is invariable among species and is composed of two covalently attached macromolecules, peptidoglycan and mycoloyl arabinogalactan (Draper, 1982; McNeil & Brennan, 1991). Interestingly in the context of the host–parasite interaction in mycobacterial diseases, it has been observed that virulent mycobacteria growing intracellularly are surrounded by a capsule, observed as an electron-transparent zone by electron microscopy (Hanks, 1961), which may be part of defence mechanisms permitting all pathogenic mycobacteria to resist being killed by phagocytic cells (Draper & Rees, 1970). More recently, it has been shown that in vitro-grown pathogenic species are also surrounded by a capsule (Fréhel et al., 1988; Paul & Beveridge, 1994). Because the outermost constituents of this protective capsule are in direct contact with host cells, they may play an important role in the phagocytosis process leading to the internalization of the pathogen. They may also determine, in part, the nature and the intensity of the host response towards the phagocytosed bacilli. As far as the chemical nature of the mycobacterial outer layer is concerned, however, information is generally scarce.

The outermost constituents of the bacilli are confined around the bacterial cells when the environment happens to be a macrophage, but may be released by actively growing mycobacteria in artificial culture media, which may also contain secreted substances. In the past few years, numerous studies have been devoted to the isolation, characterization and biological activities of the proteins derived from early-exponential-phase culture filtrates (Andersen & Brennan, 1994; Harboe, 1992; Young et al., 1992). However, little attention has been paid to extracellular polysaccharides, despite the early demonstration of the presence of polysaccharides in tuberculin material derived from the culture filtrate of the tubercle bacillus and used for skin testing (Seibert, 1949). Because of the presence of arabinogalactan, a true cell wall constituent (Draper, 1982; McNeil & Brennan, 1991), as the major antigenic component of tuberculin (Daniel, 1984), the impression prevails that the extracellular polysaccharides are derived from the autolysis of bacterial cells.

Previously, we have investigated the presence of polysaccharides in early-exponential-phase culture filtrate of M. tuberculosis devoid of somatic and cell-wall-associated compounds (Lemassu & Daffe, 1994). We showed that the extracellular material was composed of proteins and three types of neutral polysaccharides: a glucan, an arabinomannan (AM) and a mannan. We also demonstrated that the same types of polysaccharides are present at the surface of the bacterial capsule (Ortalo-Magné et al., 1995), suggesting that these polysaccharides are capsular constituents that are shed from growing cells into the culture medium. As mycobacterial species differ in their cell-wall-associated lipids, growth rate, drug susceptibility, solute permeability, antigenicity and pathogenicity, the data obtained for the tubercle bacillus may not be universal. Furthermore, we showed recently that mycobacterial species differ in their surface-exposed lipids (Ortalo-Magné et al., 1996b). In this study, we report the comparison of the carbohydrate content of the extracellular materials (ECMs) and surface-exposed materials (SXMs) of selected mycobacterial species and the chemical characterization of the major polysaccharides that these materials comprise.

METHODS

Strains and growth conditions. M. avium ATCC 13769 and TMC 1468, M. gastri W471, M. kansasi ATCC 12478, IP 890175 and IP 890370, M. phlei ATCC 11758, M. smegmatii ATCC 607 and M. tuberculosis strain Canetti (CIPT 140010059), its spontaneous rough revertant (59R, Lemassu et al., 1992) and the Canetti-like strain CIPT 140010060 were grown on Sauton's medium (Sauton, 1912) (100 ml per flask) as surface pellicles at 37 °C. Smooth transparent (SmT) and smooth domed-opaque (SmD) colonial variants of M. avium strain 485 (Fattorini et al., 1994) were grown on Middlebrook 7H9 plus glycerol for 10 d at 37 °C as described by Fattorini et al. (1994). The spontaneous reversion frequency of SmT into SmD, a common occurrence in liquid medium, was estimated as 7% by observing the colony morphology on Middlebrook 7H10 agar plates.

Production of ECM during growth. Experiments were performed with bacteria from three independent batches throughout the growth phases. Cells were harvested from 100 ml culture flasks by filtration through a Durieux filter and the corresponding extracellular materials were obtained by filtering the culture medium through a 0.2 μm sterile Filter (Nalgene). They were concentrated under vacuum to one-tenth of their original volume, dialysed, lyophilized and weighed. Cells were extensively washed with distilled water, lyophilized and weighed.

Isolation of SXMs. The pellicular growth allowed an easy harvest of cells by permitting the medium to be poured off when the pellicles remained attached to the flasks. Cells were harvested from 10–20 flasks and gently shaken (1000 r.p.m.) for 1 min with 10 g glass beads (2 g wet cells), a method known not to affect the integrity of cells (Ortalo-Magné et al., 1995). The resulting SXMs were then resuspended in distilled water (50 ml per flask) and immediately filtered through a 0.2 μm sterile Filter (Nalgene).

Fractionation of ECM and SXM. Filtrates (derived from the
extracellular medium and from the mechanical treatment of cells) were concentrated under vacuum to one-tenth of the original volumes; chloroform and methanol were then added to the filtrates to obtain a partition mixture comprising chloroform/methanol/water (3:4:3, by vol.). The organic phases were dried and weighed. The glucans were recovered as opalescent solutions by extracting the interphases several times with water (Lemassu & Daiffé, 1994). The aqueous phase and the glucan solution were concentrated and the polymers were precipitated overnight at 4 °C with 6 vols cold ethanol. The precipitates were collected after centrifugation at 14,000 g for 1 h and dissolved in distilled water. The polymers were then precipitated again with ethanol, re-centrifuged and the precipitates dialysed for 3 d against water to eliminate traces of glycerol and salts before being lyophilized and weighed.

Purification of the different polysaccharides. The polymers derived from the aqueous phases were dissolved in 50 mM NH₄HCO₃, treated twice with trypsin (2%, w/w, of the protein content) for 5 h at 37 °C and dialysed for 3 d against distilled water. The resulting material was assessed for the presence of protein, which usually did not exceed 2%. When the percentage of protein exceeded 2%, however, the treatment with trypsin was repeated. Then, the different polysaccharides were purified as previously described in detail (Lemassu & Daiffé, 1994). Briefly, Ara/Man-containing polysaccharides and Glc-rich polysaccharides were chromatographed on a column of DEAE-Trisacryl gel and the neutral fractions were rechromatographed on columns of Bio-Gel P-10 and Sephadex G-200, respectively. The elution profiles of both types of gel filtration chromatography were monitored by refractive index detection and the collected fractions were assessed for their carbohydrate content (Dische, 1962). The glycosyl composition of pooled fractions corresponding to the different chromatography peaks was determined by acid hydrolysis (2 M CF₂COOH at 110 °C for 2 h), followed by analysis of the trimethylsilylated sugar derivatives by GC.

Analytical techniques. The dialysed culture filtrates and the various chromatographic fractions were assessed for the presence of protein (Lowry method) and carbohydrate (Dische, 1962). The percentage of carbohydrate was also determined by GC using erythritol as internal standard.

Pure polysaccharides (2 mg) were per-O-methylated four times according to the method of Cucanu & Kerek (1984). Portions of the per-O-methylated products were hydrolysed with 2 M CF₂COOH at 110 °C for 2 h, reduced with NaBH₄ and acetylated. The different partially O-methylated and partially O-acetylated alditols were identified by GC-MS.

GC-MS was performed on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5989A Mass Selective Detector. The column was a 12 m HP-1 (Hewlett-Packard). Samples were injected in the Splitless mode. The oven temperature was programmed to hold at 80 °C for 1 min followed by a 15 °C min⁻¹ increase to 290 °C. The mass spectrometer was set to scan from 50 to 600 atomic mass units.

GC was performed on alditol acetates and trimethylsilyl derivatives of monosaccharides using a Girdel chromatograph (model G 30) equipped with a fused silica capillary column (25 m length x 0.22 mm i.d.) containing WCOT OV-1 (0.3 mm film thickness, Spiral). A temperature gradient of 100–280 °C at 2 °C min⁻¹ was used.

NMR spectra were obtained in D₂O with a Bruker 250 WB instrument equipped with an Aspect 3000 computer. One-dimensional spectra were obtained with 40 ° pulses and an acquisition time of 1:56 s for 13C-NMR (spectral width: 5263 Hz) and 2:73 s for 1H-NMR (spectral width: 2994 Hz) was used. The chemical shift reference used was that of tetramethylsilane.

RESULTS

Production of ECM during growth

Growth curves and measurement of ECM derived from 100 ml culture filtrates and produced during the different growth phases of the various mycobacterial strains were performed on bacterial cells which were extensively washed, dried and weighed, as all the mycobacterial species used in the present study formed clumps when

![Fig. 1. Production of ECM (●) during the growth of M. tuberculosis (a), M. smegmatis (b) and M. avium (c). Cells were harvested, extensively washed, lyophilized and weighed. Cell dry weight (○) is plotted on a log scale. ECMs were obtained by concentration of 100 ml culture filtrates, followed by extensive dialysis and lyophilization as described in Methods. Results represent the arithmetic mean of three determinations.](https://www.microbiologyresearch.org)
Table 1. Composition of ECM and SXM from different mycobacterial species

<table>
<thead>
<tr>
<th>Strain</th>
<th>Component</th>
<th>Yield*</th>
<th>Carbohydrate (%)</th>
<th>Protein (%)</th>
<th>Sugar composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glc</td>
</tr>
<tr>
<td>M. gastri</td>
<td>ECM</td>
<td>1.0±0.1</td>
<td>71</td>
<td>28</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>SXM</td>
<td>95</td>
<td></td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>ECM</td>
<td>16.0±0.2</td>
<td>84</td>
<td>16</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>SXM</td>
<td>92</td>
<td></td>
<td>7</td>
<td>87</td>
</tr>
<tr>
<td>M. avium</td>
<td>ECM</td>
<td>0.9±0.1</td>
<td>48</td>
<td>51</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>SXM</td>
<td>86</td>
<td></td>
<td>14</td>
<td>94</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>ECM</td>
<td>2.5±1.0</td>
<td>26</td>
<td>74</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>SXM</td>
<td>27</td>
<td></td>
<td>73</td>
<td>28</td>
</tr>
<tr>
<td>M. phlei</td>
<td>ECM</td>
<td>3.0±0.5</td>
<td>21</td>
<td>79</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>SXM</td>
<td>17</td>
<td></td>
<td>83</td>
<td>5</td>
</tr>
</tbody>
</table>

*Yield is expressed as mg of materials obtained from early-exponential-phase culture filtrates (as described in Methods) per 100 mg of the corresponding mycobacterial dry weight±SD.
†The relative amount of Xyl may vary significantly according to the batches and the strains examined (see text).

Growing both as surface pellicles and under stirring. Furthermore, the conventional addition of detergent to the cultures to prevent the formation of clumps during growth, was not suitable, as this method releases additional materials from the cells into the extracellular medium. Thus, the culture filtrates corresponding to the various growth phases of a slow grower (M. avium), a rapid grower (M. smegmatis) and M. tuberculosis (used as reference) were collected, submitted to ethanol precipitation, dialysed and weighed to yield the crude unfractionated ECM. As illustrated in Fig. 1, two types of curves were obtained: while the production of ECM followed the growth curve of M. tuberculosis (Fig. 1a), an invariable and low level of ECM was observed for both M. avium and M. smegmatis (Fig. 1b,c). To compare the ECM production of the different species to that of M. tuberculosis (Lemassu & Daffé, 1994), the ECMs of the different strains were collected from early-exponential-phase growth culture filtrates. As shown in Table 1, the ECMs of M. avium, M. gastri, M. phlei and M. smegmatis corresponded to only 1-3% of the dried bacterial mass. In contrast, high levels of ECM were produced by M. kansasii, comparable to that previously found for M. tuberculosis (Lemassu & Daffé, 1994). These data demonstrated the existence of high level and low level producers of ECM among mycobacteria.

**Chemical nature of ECM and SXM**

Small amounts of SXM were recovered from the bacilli by mechanical treatment with glass beads. They represented 10-14% of the mass of the corresponding ECM. When chloroform/methanol/water partition was applied prior to ethanol precipitation, the organic phases derived from the extraction of the culture filtrates of the different mycobacterial strains contained little (≤ 1%) or no lipids. Small amounts of lipids (1-5%) were present in the organic phases derived from the partition of SXM. The unfractionated ECM and SXM of the various mycobacterial strains were composed mainly (≥ 95%) of carbohydrates and proteins. According to the carbohydrate and protein contents of ECM and SXM (Table 1), the mycobacterial species examined may be divided into three groups: the materials derived from M. gastri and M. kansasii contained much more carbohydrate (up to 95%) than protein, whereas the reverse was true for M. phlei and M. smegmatis. M. avium was unique in that the examined strain contained carbohydrate-rich SXM and ECM composed of equal amounts of protein and carbohydrate (Table 1).

The monosaccharide composition of ECM and SXM, determined by acid hydrolysis of the polymers, was found to be qualitatively invariant during the exponential growth phase of all the examined strains of M. kansasii. The ECM and SXM of M. kansasii and those of M. smegmatis were of similar composition but distinguished from each other by the presence of Xyl in the materials derived from M. smegmatis. Although the ECM and SXM of M. gastri were composed of the same sugar constituents, much less Ara and Man were present in SXM (Table 1).

In contrast to these mycobacterial species, the sugar composition of the ECM of M. avium and M. phlei differed from that of the SXM. The glycosyl composition of the ECM of M. avium varied between batches. Xyl was detected in the acid hydrolysis products of the ECM in most of the batches of the two examined strains of M.
avium but in some other batches this sugar was not detected at all. The ECM from M. avium differed also from the other ECM in its content of Man, which was the major sugar in the acid hydrolysis of all the ECM derived from the different batches of the two strains of M. avium. In the case of M. phlei, a tiny amount of Xyl was present in the ECM, whereas this sugar represented the major constituent of the SXM. The two species were also unusual in the glycosyl composition of their SXM which was invariably found to be devoid of Ara (Table 1). It is interesting to note that the acid hydrolysis products of the ECM and SXM derived from the SmT and SmD variants of M. avium, the two well-studied morphotypes, showed similar glycosyl compositions, including the absence of Ara in SXM (data not shown).

**Purification and characterization of the major polysaccharides**

Over 90% (by wt) of the water-soluble Ara/Man-containing polysaccharides from the extracellular and surface-exposed materials of the different mycobacterial species examined were neutral compounds, since they were eluted from the DEAE-Trisacryl column with the buffer containing no NaCl. Chromatography of these neutral polysaccharides on Bio-Gel P-10 columns using 1% (v/v) acetic acid in water as eluant gave qualitatively similar profiles for the various species, leading to the isolation of two major fractions (Fig. 2a). The first major peak (Ve = 65 ml), exhibiting an apparent molecular mass of approximately 13 kDa (using dextrans as reference compounds), contained AMs (Lemassu & Daffé, 1994). The second major fraction (Ve = 90 ml) consisted of mannans with an apparent molecular mass of 4 kDa. The minor peak eluted in a position corresponding to the void volume (Voe, molecular mass > 20 kDa); acid hydrolysis showed the presence of Ara, Man and Glc. Based on our previous results (Lemassu & Daffé, 1994), it was concluded that the latter chromatographic peak contained a mixture of AMs and mannans, and the small portion of glucans that had been dragged in the aqueous phase during the partition experiment.

The glucans from both sources were not retained on the anion-exchange columns. When analysed in Sephadex G-200 columns (Fig. 2b), the polysaccharides were eluted at a position (Ve = 70 ml) corresponding to an apparent molecular mass of 120 kDa.

The Xyl-containing carbohydrates, eluted in the last fraction of the Bio-Gel P-10 column (Ve = 130 ml) were not investigated further.

**Structural features of the major polysaccharides**

Aliquots of the purified AMs from ECM and SXM were per-O-methylated, hydrolysed, reduced, O-acetylated and analysed by GC-MS. The arabian segments consisted of terminal, 2-, 3,5- and 5-linked Ara residues. The mannan segments contained terminal, 6- and 2,6-linked pyranosides since all the mannansyl residues, after per-O-methylation, contained a methoxyl group located on carbon 4. All the AMs, except that of M. smegmatis, also contained 2-linked Manp. In addition, the 13C-NMR spectra of the purified AMs (data not shown) were superimposable on that of M. tuberculosis (Lemassu & Daffé, 1994), suggesting that the different purified AMs may share the major structural feature that consists of a \([\rightarrow 6]\)Manp(1\(\rightarrow\)) mannan core, substituted by arabian segments composed mainly of \([\rightarrow 5]\)Arap(1\(\rightarrow\)) (Lemassu & Daffé, 1994; Misaki et al., 1977). No signal attributable to acyl functions, between 0 and 30 p.p.m., was observed in the 13C-NMR spectra. Likewise, fatty acyl proton resonance signals (observable between 0 and 3 p.p.m.) were absent from the 1H-NMR spectra. These data demonstrated that the polysaccharides under study were devoid of fatty acyl substituents and are consistent with the identification of Man at the reducing end of the corresponding molecules of M. tuberculosis (Ortalo-Magné et al., 1996a).

To address the question of the structure of the non-reducing ends of the arabian segments of the various...
Table 2. Glycosyl linkage composition of the AMs of M. avium and M. smegmatis

<table>
<thead>
<tr>
<th>Glycosyl residues</th>
<th>Glycosyl linkage composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. avium</td>
</tr>
<tr>
<td>t-Ara$_r$</td>
<td>3</td>
</tr>
<tr>
<td>2-Ara$_r$</td>
<td>7</td>
</tr>
<tr>
<td>5-Ara$_r$</td>
<td>40</td>
</tr>
<tr>
<td>3,5-Ara$_r$</td>
<td>7</td>
</tr>
<tr>
<td>t-Man$_p$</td>
<td>22</td>
</tr>
<tr>
<td>2-Man$_p$</td>
<td>12</td>
</tr>
<tr>
<td>6-Man$_p$</td>
<td>2</td>
</tr>
<tr>
<td>2,6-Man$_p$</td>
<td>6</td>
</tr>
</tbody>
</table>

AMs, the glycosyl linkage composition of the polysaccharides was examined. In mycobacterial arabinogalactans (Daffé et al., 1990, 1993) and uncapped lipoarabinomannan (Chatterjee et al., 1991), the molar ratio of terminal β-Ara$_r$ to 3,5-linked Ara$_r$ was always approximately 1. The AM of M. smegmatis, but not that derived from the ECM of M. avium, (Table 2) fulfill this criterion, suggesting some differences in the terminal region of their arabinan segments. The combined evidence of small amounts of terminal Ara$_r$ (as compared to 3,5-linked Ara$_r$) and the excess of terminal Man$_p$ (as compared to 2,6-linked Man$_p$) strongly suggests the substitution of the non-reducing end of the arabinan segment of the AM of M. avium by either a terminal Man or an oligomannoside. This hypothesis is further substantiated by the occurrence of 2-linked Man$_p$ in the AM of M. avium, but not in that of M. smegmatis (Table 2). These data were in agreement with the finding that 2-linked Man$_p$ residues are part of the oligomannosyls that substitute the arabinan termini of the so-called capped lipoarabinomannans (Chatterjee et al., 1992). Analysis of the glycosyl linkage composition of the per-O-methylated AMs of M. kansasii and M. gastri (data not shown) led to the conclusion that these molecules were as extensively capped as those of M. avium and M. tuberculosis (Lemassu & Daffé, 1994; Ortalo-Magné et al., 1995). However, the extent of capping with mannosyl residues may vary according to the strains and the growth phase of the same strain.

The glucans purified from the ECM and SXM of the different species were also per-O-methylated and their partially O-methylated, partially O-acetylated alditol derivatives were analysed by GC-MS. It appeared that the vast majority of the glucosyl residues were either 4-linked Glc$_p$ (or 5-linked Glc$_r$), as previously found for those of M. tuberculosis (Lemassu & Daffé, 1994; Ortalo-Magné et al., 1995); some branched 4,6- (or 5,6-linked) glucosyl residues were also detected. To address the question of the anomic configuration and of the ring form of the glucosyl units, the purified glucans were subjected to $^{13}$C-NMR analysis. The spectra were superimposable on that of M. tuberculosis (Ortalo-Magné et al., 1995). The anomic resonance signal was seen at 101 p.p.m. which can only result from the resonances of C-1s of $\alpha$-Glc$_p$, the C-1 resonances of $\beta$-Glc$_p$ and of $\alpha$- and $\beta$-Glc$_r$ being expected at lower field (104–110 p.p.m., Bradbury & Jenkins, 1984). No fatty acyl proton and carbon signal resonance was observed in the NMR spectra. These results supported a conserved structure for the neutral, non-lipidated mycobacterial outer layer glucans, based on a linear \((\rightarrow 4)-\alpha-D-GlcP(1 \rightarrow )\).

Extracellular materials and colony morphology

Previous studies have demonstrated that the colony morphology of some strains of V. cholerae may be correlated with the production of large amounts of extracellular polysaccharides (Johnson et al., 1992), whereas in Clavibacter michiganense, the molecular masses of these polysaccharides may determine the texture of the colonies (Henningson & Gudmestad, 1992). In mycobacteria, however, no such data has been published. Thus, we compared the production of extracellular materials of smooth and rough colony-forming strains of M. tuberculosis and M. kansasii which consist mainly of polysaccharides (see Lemassu & Daffé, 1994, and Table 1, respectively). No obvious correlation was observed between the amount of ECM and the colony morphology. The smooth strain 60 of M. tuberculosis, a Canetti-like strain, produced no more ECM than the spontaneous rough revertant of the Canetti strain, 59R [17–20 mg ECM (100 mg bacteria)$^{-1}$] although strain Canetti itself produced more [about 33 mg ECM (100 mg bacteria)$^{-1}$]. A smooth strain of M. kansasii, ATCC 12478, produced only slightly more ECM [24 mg (100 mg bacteria)$^{-1}$] than two rough strains, IP 175 and IP 370, of the same species [14–18 mg (100 mg bacteria)$^{-1}$]. To determine the possible influence of the structural features of the extracellular polysaccharides on the colony morphology of the strains, the major extracellular polysaccharides of the isogenic strains 59 and 59R of M. tuberculosis, i.e. the glucans, AMs and mannans, were purified from the culture filtrates and their structural features were determined as described above. Again, no difference was noted between the analysed compounds. The polysaccharides exhibited the same apparent molecular masses on gel filtration columns, gave superimposable NMR spectra and contained similar amounts of the different glycosyl residues. It followed then that the structures and amounts of the major mycobacterial extracellular polysaccharides are similar in both smooth and rough colony-forming strains.

DISCUSSION

The chemical definition of the mycobacterial cell envelopes is central to understanding the pathogenesis of the diseases caused by these bacteria. One important question is whether there is any correlation between the cell envelope composition and the pathogenicity and/or the
growth rate of the various mycobacterial species. Accordingly, we compared the chemical nature of the outermost constituents of five pathogenic and non-pathogenic mycobacterial species.

Based on the relative amounts and the kinetics of production of ECM during growth, the mycobacterial species examined so far are not uniform and may be divided into two main groups. The member of the first group is the pathogen M. kansasii, (Wayne & Kubica, 1986). This species produces a high level of carbohydrate-rich ECM, whose polysaccharide composition is quantitatively and qualitatively similar to that of SXM. The second group comprises the pathogenic species M. avium and the non-pathogenic species M. gastri, M. phlei and M. smegmatis. They produce small amounts of ECM, whose carbohydrate composition may be qualitatively different from that found on their cell surface. A subdivision of this latter group into carbohydrate- and protein-rich SXM was also possible. M. phlei and M. smegmatis contain protein-rich SXM, while M. avium and M. gastri contain carbohydrate-rich SXM, a classification that coincides with the difference in the growth rate of the species. These data suggest that the differences in the production and composition of ECM and SXM between mycobacterial species probably reflect differences in their cell envelope architecture. In that connection, it is interesting to note that differences have been demonstrated recently in the location of the various classes of lipids on the mycobacterial cell surfaces (Ortolan-Magné et al., 1996b). The relative contribution of ECM and SXM components to mycobacterial cell envelopes warrants further investigation. Nevertheless, it has to be noted that Man receptors have been shown to be involved in the phagocytosis of M. avium (Bermudez et al., 1991). The failure to detect AMs among the SXM of M. avium suggests that the mycobacterial components that interact with the Man receptors during phagocytosis may not be the postulated lipoarabinomannans (Schlesinger et al., 1994). It is also noteworthy that the ECM of M. avium and M. phlei, but not the SXM of the same species, contained Ara (presumably from AMs), suggesting the existence of true secreted polysaccharides (composed at least of AMs), which, in addition to those shed from the outermost surface of the cells, will compose the different ECMs.

The polysaccharides of the culture filtrates and those exposed on the cell surface consist of high molecular mass glycogen-like glucans, lipid-free AMs and mannans, structurally similar to those previously characterized in the tubercle bacillus (Lemassu & Daffe, 1994; Ortolan-Magné et al., 1995). The only structural difference observed between the polysaccharides of the various species resides in the absence of additional mannosyl residues attached to the non-reducing termini of the arabinan segments of the AMs of M. smegmatis. As this species, in contrast to the other strains examined herein, is a rapid grower, the above structural difference is in agreement with the proposal stating that capping of the arabinan termini with mannosyl residues may be more a feature of rapid growth than of virulence (Khoo et al., 1995; Prinzis et al., 1993).

The polysaccharides derived from both ECM and SXM of the mycobacterial species examined herein were almost exclusively neutral substances that were not retained on the ion-exchange column. This observation raises the question of the nature of the compounds that strongly react with ruthenium red at the cell surface of mycobacteria (Rastogi et al., 1986). This staining method has been used to demonstrate the existence of a true third 'polysaccharide-rich outer layer' (POL) in mycobacteria, as opposed to the two-layered mycobacterial cell wall (Draper, 1982; Köhler, 1984). Alternatively, the specificity of the stain may be questioned.

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