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Isolation in High Frequency of Rough Variants of *Mycobacterium intracellulare* Lacking C-Mycoside Glycopeptidolipid Antigens

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Rough variants of serovars from the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex were isolated in high frequency from pellicle growth of the wild-type strains. Rough morphology could be correlated with the lack of an outer cell wall sheath and its constituent C-mycoside glycopeptidolipids of both the serologically active and inactive types.

It has recently emerged that the surface antigens which characterize member serovars of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS) complex are polar C-mycoside glycopeptidolipids (GPLs), which are essentially more glycosylated versions of the apolar C-mycosides (3, 5). Moreover, we have revealed that serological variance among members of the MAIS complex is due to subtle differences in the structures of the short oligosaccharides which embellish the communal apolar C-mycoside structure (2a, 4). In addition, it is known that an extracellular sheath (originally called the L_1 layer by Barksdale and Kim [1, 11]) produced by MAIS organisms is predominately composed of the GPL antigens (2).

When freshly isolated, most strains of the MAIS complex are of smooth morphology and either mostly transparent or opaque (12). However, we have noticed that pure cultures propagated in the laboratory under certain conditions display rough morphology. In the realization that rough mutants of MAIS complex organisms, perhaps devoid of GPL sugar determinants, would be invaluable in exploring the biosynthesis, genetics, and physiological function of the GPLs, we have sought the means of obtaining spontaneous rough mutants in high frequency. In this report, we describe the facile isolation of such mutants and their characteristics vis-a-vis the GPL antigens and the outer cell wall sheath.

When *M. intracellulare* (serovar 20) was repeatedly plated onto 7H11 agar (6), smooth colonies prevailed (Fig. 1A). However, it was noticed that pellicle growth resulted in a dramatic enrichment of rough-colony formation. Optimal yields were obtained in the following manner. A mass of cells was removed from agar plates and placed on the inner walls of screw-capped glass tubes (150 by 25 mm) containing 10 ml of 7H11 liquid medium (6). The tubes were maintained at 37°C with the cap loosened and at a slight (20°) angle, thus locating the mycobacterial mass at the broth interface. After about 3 weeks, slight pellicle growth appeared on the surface of the broth, which was then subcultured in fresh 7H11 broth. The presence of Tween 80 in the medium did not hinder pellicle formation. Repeated (2 to 3×) subculturing eventually resulted in a culture containing only rough morphology (Fig. 1B), and repeated plating on 7H11 agar established that these variants were stable. To obtain a completely rough culture in less time, an isolated rough colony could be picked directly from plates of the first pellicle and then subcultured on liquid medium.

A possible explanation for the success of this selective procedure is that a natural colony contains only a few rough mutants whose growth may be subdued by an overabundance of smooth-colony-forming cells. However, with the introduction of conditions for surface growth, these constraints are somehow lifted, perhaps owing to the hydrophobicity of the mycobacterium, resulting in better growth of the rough mutants. Such a relationship probably exists among certain gram-negative bacteria where rough mutants are not readily detected in a culture of the parent wild strain, owing to diminished vitality (13).

Rough colonies were obtained from several members of the MAIS complex, harvested,

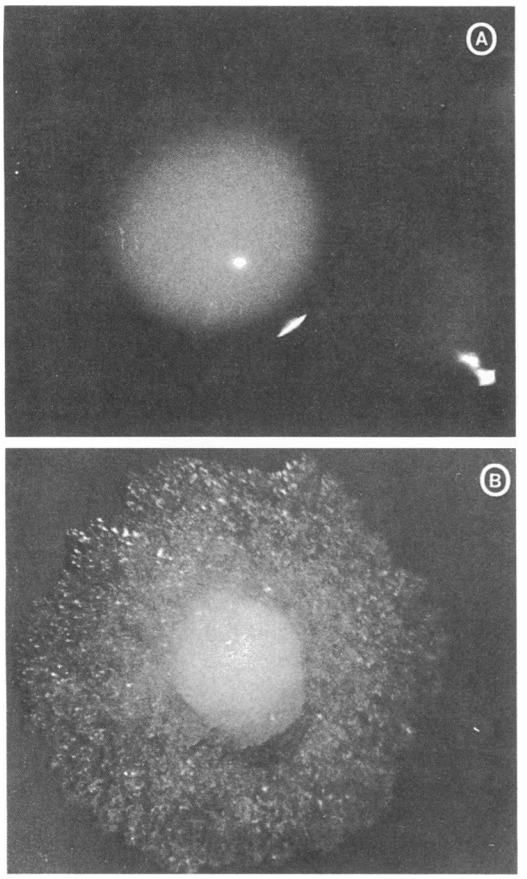

FIG. 1. Examples of (A) smooth (opaque) and (B) rough colonies of *M. intracellulare* serovar 20. Magnification, ×40.

washed, dried in vacuo over P_2O_5, and extracted for lipids with $CHCl_3$:CH_3OH (2:1) (5). Lipids from the smooth-colony variant of the homologous serovars were derived likewise. Thin-layer chromatography of the total lipid extracts in solvents designed to resolve both the apolar and polar GPLs showed the absence of both classes from the rough variants. The effects of the acquisition of rough morphology on the polar GPLs of serovar 20 are shown in Fig. 2A. The smooth variant contains its normal complement of four polar GPLs; these have in common a tetrasaccharide attached to the peptide moiety of the C-mycoside core but differ in the number of *O*-acetyl substituents (2). Likewise, chromatography in a solvent less polar than that used for Fig. 2A (e.g., $CHCl_3$:CH_3OH [11:1]) showed that the rough variant was also devoid of the apolar GPLs (data not shown). However, the normal complement of other lipids, among them phospholipids and neutral lipids, was retained in the rough variants. Indeed, loss of GPLs and the acquisition of rough morphology were the only observed alterations in phenotype and presumably are complementary events.

We have previously observed that the isolated cell wall sheath of the smooth variant of MAIS complex serovars consisted in large measure of the polar GPLs (2). Hence, one would expect the rough variant to be deficient in these so-called integuments (11). Therefore, harvested cells of both the smooth and rough variants of serovar 20 were directly mounted on Formvar carbon-coated grids, negatively stained, and examined by electron microscopy (Fig. 3). The cells of the smooth-colony variants were characterized by associated and adjacent fibrillar,

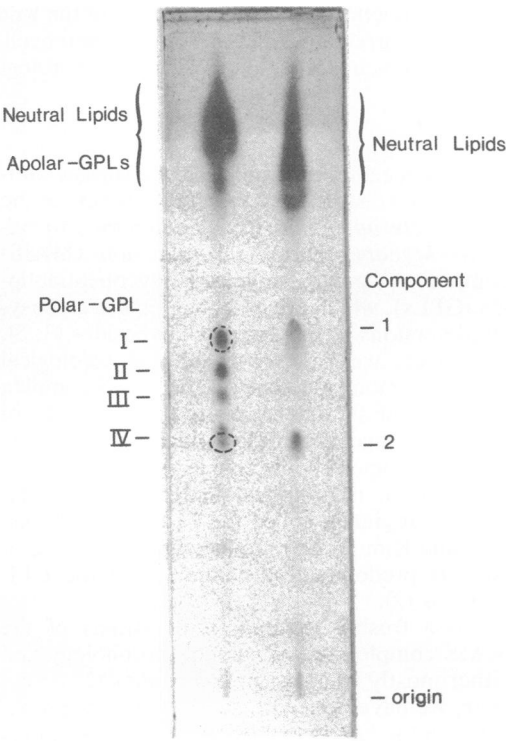

FIG. 2. Thin-layer chromatography of $CHCl_3$:CH_3OH (2:1)-extracted lipids from the smooth and rough variants of *M. intracellulare* serovar 20 in $CHCl_3$:CH_3OH:H_2O (60:12:1). (A) Lipid (ca. 100 μg) from smooth-colony variant; (B) lipid (ca. 100 μg) from rough-colony variant. The structures of polar GPLs I through IV have been described (2). The GPLs produce a characteristic yellow-gold color with an orcinol-H_2SO_4 spray. Components 1 and 2, which are probably phospholipids, stain pink and purple, respectively. These are particularly prominent in rough mutants, owing to the absence of polar GPLs, although they are also present in the smooth variants (broken-line circles) underlying the more prominent polar GPLs.

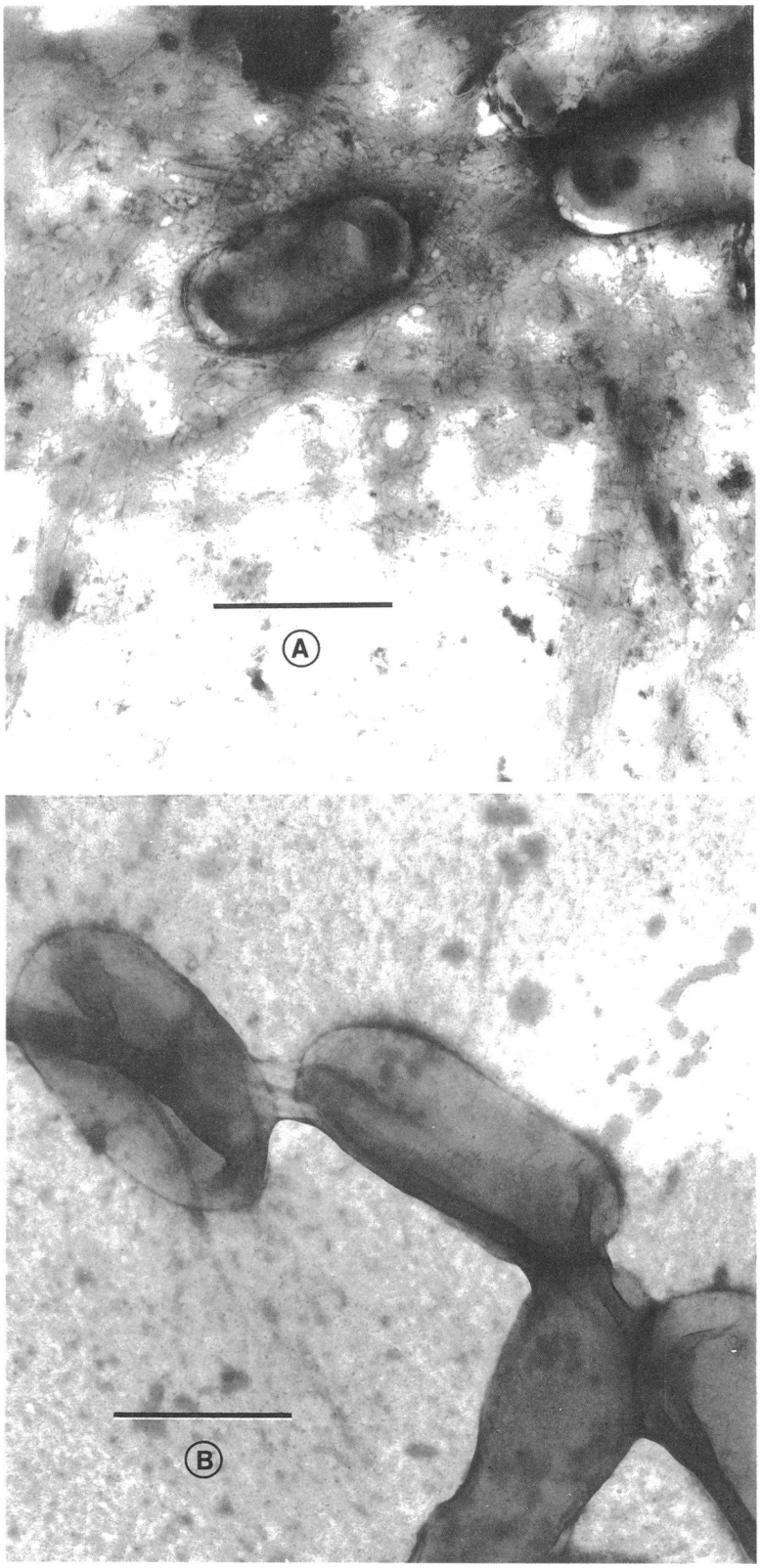

FIG. 3. Electron micrographs of the harvested and washed smooth (A) and rough (B) variants of *M. intracellulare* serovar 20. Cells were directly mounted on Formvar carbon-coated grids, treated with 2% glutaraldehyde for 30 min, washed with double-distilled water, and stained with 1.5% phosphotungstic acid. Grids were examined in an AEI Corinth 500 transmission electron micrograph operating at 60 kV. Bar, 1.0 μm.

transparent material, consisting in large part of parallel fibrils. In appearance it was identical to the parallel fibrils found associated with *Mycobacterium lepraemurium* within tissue (8) and to the material derived from *M. avium* grown in static fashion (7). They were also identical in ultrastructure to the isolated superficial cell wall sheath described previously by us (2). Rough variants, on the other hand, were devoid of the fibrillar filamentous material (Fig. 3B), although there was evidence for the emergence of an unidentified amorphous substance.

This close correlation between rough morphology, absence of C-mycoside GPLs, and lack of a superficial cell wall sheath probably underlies observations made by Fregnan et al. some 20 years ago (9). A rough variant which spontaneously developed after 2 months of incubation from a culture of an unclassified scotochromogenic *Mycobacterium* strain was initially observed as a thin surface growth. Subsequent transfers produced a culture totally devoid of a "mycoside D" that had been present in the smooth-colony-forming parental strain. There is little doubt that "mycoside D" was a mixture of the polar and apolar C-mycoside GPLs recently defined in detail by us (10).

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