Expression of the Core Lipopeptide of the Glycopeptidolipid Surface Antigens in Rough Mutants of Mycobacterium avium*

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Toward studying the genetics, biosynthesis, and roles in the pathogenesis of the dominant surface glycopeptidolipid antigens of Mycobacterium avium, rough colony variants of M. avium serovar 2 were picked, cultured in quantity, and their lipid compositions determined. Two broad classes of (Rg) variants, Rg-3 and Rg-4, were devoid of glycopeptidolipids or any more elemental structures and thus were similar to those described previously. Two others, Rg-0 and Rg-1, each contained two novel lipopeptides, devoid of any of the carbohydrate substituents that confer serotypic activity on the glycopeptidolipids. The application of gas chromatography, fast atom bombardment-mass spectrometry and "H NMR to lipopeptide I established the structure C₄₆₋₇₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋⁻

Disseminated infections caused by organisms of the Mycobacterium avium complex are among the most common opportunistic infections in patients at advanced stages of human immunodeficiency virus infections (1). Because of convincing evidence that M. avium bacteremia contributes significantly to morbidity and mortality of patients, there is now considerable interest in developing innovative chemophylactic strategies for organisms that are extremely difficult to eradicate (2). The cell envelope of members of the M. avium complex possesses glycolipid antigens, the glycopeptidolipids (GPLs), which are confined to this set of mycobacteria and are of use in their differentiation from all others (3). The GPLs are located on the surface and are associated with the capsular-like matrix that surrounds the bacillus and forms an electron transparent zone in phagocytic vesicles (3-6). They are thought to suppress lymphocyte blastogenesis (7, 8), a phenomenon observed in M. avium complex infections (9, 10). However, their actual contribution to the pathogenesis of M. avium infections and the intracellular survival of bacilli has been questioned (11, 12), since both the virulent smooth (SmT) and avirulent smooth domed (SmD) colony forms express GPLs, whereas those rough (Rg) variants isolated to date have variously been described as virulent (13) or avirulent (14) and are completely devoid of the surface antigens (15). To resolve obvious confusion and paradoxes, there is a pressing need to develop a range of genetically and phenotypically defined mutants. Such mutants should also lead to an understanding of the pathways for GPL biosynthesis, the underlying genetics responsible for GPL biosynthesis, and the roles of what are copious products in bacterial invasiveness and persistence.

Two forms of glycopeptidolipids are present in the M. avium complex, the ssGPL and naGPL (3). Both the ss- and naGPLs have the same basic tripeptide-amino alcohol "core" component of the glycopeptidolipids of the M. avium complex, and thus the Rg-0 and Rg-1 variants represent a form of "deep rough" maturation in M. avium. Separately, we report that these rough variants of M. avium differ genetically from the smooth, virulent form by major deletions of portions of the genes responsible for glycopeptidolipid synthesis (Belisle, J. T., Kweekiewicz, K., Brennan, P. J., Jacobs, W. R., Jr., and Inamine, J. M. (1993) J. Biol. Chem. 268, 10517-10523). The isolation of different sets of spontaneous mutants of M. avium differentially defective in the capacity to synthesize glycopeptidolipids provides the means to explore their biosynthesis and roles in opportunistic pathogenesis.

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1 The abbreviations used are: GPL(s), glycopeptidolipids; ns-, nonspecific; ss-, serovar-specific; Rg, rough; SmD, smooth domed; SmT, smooth transparent; TLC, thin layer chromatography; GC, gas chromatography; MS, mass spectrometry; PAB, fast atom bombardment; PBS, phosphate-buffered saline.
negative, lipopeptide-negative, and the present new mutants that are GPL-negative, lipopeptide-positive. The results further demonstrate that the initial step in GPL biosynthesis is the formation of a lipopeptide core, which obviously is the parent of both the ns- and ssGPLs. A description of the genonomic differences that define these two phenotypes is presented separately (18).

EXPERIMENTAL PROCEDURES

Growth of M. avium and Isolation of Morphological Variants—M. avium serovar 2 (strain 2151) was originally obtained from the spumum of an individual with pulmonary mycobacterioses. It was initially plated on 7H11 agar (19), and, after 3 weeks of incubation at 37°C, three morphological variants (SmD, SmT, and a Rg isolate termed Rg-0 (13, 14)) were picked and subcultured on 7H11 agar. Homogeneous colony morphology was ensured by subculturing each isolate a total of three times. Colonies from the third passage of these strains were scraped, placed in sterile PBS with 0.05% Tween and stirred overnight to generate homogeneous suspensions which were stored as stocks at −70°C. The rough Rg-1, Rg-3, and Rg-4 isolates, single colonies that did not differ in appearance, were picked from subcultures of the SmD isolate plated on 7H11 agar. They were purified and stored as described above. Large amounts of harvest were obtained by spreading stocks of the SmD, Rg-0, Rg-1, Rg-3, and Rg-4 variants on 7H11 agar plates (15 × 150 mm) at a concentration of 4.0–4.5 × 10^7 colony-forming units/plate and incubating at 37°C. The formation of a lipopeptide core, which obviously is the parent of both the ns- and ssGPLs, was then examined before harvesting and determined to be consistently smooth domed or rough, as described (13, 14). Cells were harvested by scraping, suspended in PBS, autoclaved at 80°C, and lyophilized.

Purification of Lipopeptides 1 and 2—To determine the content of lipids in isolates, lyophilized cells were extracted with CHCl₃-CH₂OH (21) (20). The total washed lipid fractions (21) were hydrolyzed with 0.1 n NaOH at room temperature to select for alkali-stable lipids (20) and subjected to TLC in mixtures of CHCl₃ and CH₂OH on sheets of precoated Silica Gel 60 (Merck) as described (20). To isolate appreciable quantities of lipopeptide for structural characterization, 0.01 g of lyophilized M. avium Rg-1 cells were extracted as described above. The extraction yielded 235 mg of total lipid, and subsequent alkaline hydrolysis of this resulted in 200 mg of alkali-stable lipid. This lipid was dissolved in CHCl₃ and applied to a column (1.5 × 20 cm) of Florisil (60–106 mesh; Sigma) equilibrated in CHCl₃. The column was irrigated with 20 ml each of CHCl₃, CH₂OH, Lipid (30 mg) from the 10% CH₂OH fraction was applied to five 20 × 20 cm glass-backed TLC plates (Silica Gel 60 A, 0.25 mm, Whatman) that were developed six times in CHCl₃-CH₂OH (1:12:1). The major lipid bands were observed by spraying with a fine mistsolution of Silica Gel or by visualizing the plates. These lipids were recovered by extraction with CHCl₃-CH₂OH (21), washed by partitioning with water, redissolved in CHCl₃-CH₂OH (9:1), and passed through an Acrodisc CR, 0.2-μm filter (Gelman Sciences, Ann Arbor, MI) to remove remaining Silica Gel. The ssGPL of smooth variants of M. avium serovar 2 was obtained as described (22).

Analytical Methods—To examine the glycolyx content of lipids, they were hydrolyzed with 2 M CF₃COOH, and the resulting free sugars converted to alditol acetates and resolved on a DB 23 fused silica capillary column (J&W Scientific, Folsom, CA) as described (23). The amino acid composition of lipids was analyzed by GC of the N₁(O)-heptafluorobutyl oxybutyl ester derivatives as described (24). To establish the enantiomeric form of amino acids, R₁(-)isobutanol (M drunk) was used in the derivatization (25). The N₁(O)-heptafluorobutyl oxybutyl ester derivatives were separated on an HP-1 fused silica capillary column (Hewlett-Packard) as described (26).

Identification of a New Lipopeptide in Some Rg Variants—The collection of spontaneous Rg variants was examined for differences in lipid content, with a specific focus on what might be truncated forms of the basic GPL structure. Alkali-stable lipid fractions from harvests of M. avium SmD and the four rough variants (Rg-0, Rg-1, Rg-3, and Rg-4) were examined by TLC (Fig. 1). Our conjecture that all Rg variants are not identical proved to be correct in that the Rg-0 and Rg-1 variants contained two unique lipids that were absent from Rg-3 and Rg-4. Although the mobility of these two new lipids was similar to that of the nsGPL, the color produced by them in response to the spray was yellowish brown compared with the bright golden yellow color reflective of the 6-deoxyhexoses within the GPLs (23). This simple observation suggested that the new lipids in Rg-0 and Rg-1 were devoid of the usual sugar components of the GPLs. The total population of alkali-stable lipids from each isolate was hydrolyzed under conditions appropriate to sugar and amino acid cleavage, derivatized, and examined by
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**FIG. 1.** Thin layer chromatography of alkaline-stable lipids from morphological variants of *M. avium* serovar 2 strain 2151. Lane 1, SmD; lane 2, Rg-0; lane 3, Rg-1; lane 4, Rg-3; lane 5, Rg-4. The thin layer plate was developed three times in CHCl₃-CH₃OH (12:1) and sprayed with 10% H₂SO₄ in ethanol. About 80 µg of lipid was applied to each lane. The prominent component in Rg-3 and Rg-4 (lanes 4 and 5) and also evident in the other variants is a mixture of free fatty acids.

GC; the products from the SmD variant were included for comparison (Fig. 2). The lipids from Rg-0, Rg-1, Rg-3, and Rg-4 lacked the sugars 2,3-di-O-Me-fucose, rhamnose, 6-deoxytalose, and 3,4-di-O-Me-rhamnose, which are present in the combined GPL peptides of the smooth variants of *M. avium* serovar 2. On the other hand, alkaline-stable lipids from the Rg-0 and Rg-1 variants did contain the characteristic amino acids and amino alcohol of the ss- and nsGPLs, whereas those from Rg-3 and Rg-4 were devoid of these amino compounds. These results clearly demonstrated that two distinct types of spontaneous Rg mutants of *M. avium* had been isolated. One, represented by Rg-3 and Rg-4, was devoid of any elements of the GPLs and corresponded to those described previously (15), whereas the other, typified by Rg-0 and Rg-1, represented a new class of rough *M. avium* variants and obviously expressed vestiges of the GPL structure in the form, apparently, of the nonglycosylated lipopeptide core.

**Chemical Characterization of the Lipopeptides of *M. avium* Rg-1—**To isolate the putative natural lipopeptide core, total alkal-stable lipid from *M. avium* Rg-1 was applied to a column of Florisil and eluted with increasing concentrations of CH₃OH in CHCl₃. Examination of the eluates by TLC and by GC for amino acid/amino alcohol composition demonstrated that the lipopeptides appeared mostly in the 10% CH₃OH in CHCl₃ eluate. Preparative TLC of this fraction allowed recovery of sizable quantities of two new lipopeptides, lipopeptide I (4.2 mg) and lipopeptide II (5.7 mg) (Fig. 3). Thus, these lipopeptides combined represent 3-4% of the total cell weight, comparable to GPLs in smooth variants (33). GC of the amino acids/amino alcohol of lipopeptide I and II as their heptafluorobutyl isobutyl and N₃(O)-heptafluorobutyl tyryl Rₜ-isobutyl derivatives demonstrated that both contained Phe, Thr, Ala, and alaninol. ¹H NMR of per-O-acetylated lipopeptide II revealed four protons resonating as doublets at δ 7.55, δ 6.98, δ 6.65, and δ 6.25 ppm, which

**FIG. 2.** Examination of the glycosyl and amino acid content of the alkaline-stable lipid fraction from morphological variants of *M. avium* serovar 2 strain 2151. Panel A, GC of alditol acetates. The temperature program involved an increase from 80 to 160 °C at 30 °C/min followed by 2 °C/min to 180 °C and then 8 °C/min to 240 °C, which was held for 10 min. IS, an internal standard of 6-O-Me-galactitol acetate. 6d-Tal, 6-deoxytalose. Panel B, GC of N₃(O)-heptafluorobutyl isobutyl esters. The temperature program involved 85 °C for 2 min, an increase at 8 °C/min to 280 °C which was then held for 8 min. IS, internal standard of derivatized α-amino adipic acid. The asterisk represents a product of the derivatization procedure and could not be identified by MS.

**FIG. 3.** TLC of the purified lipopeptides from the Rg-1 variant of *M. avium* strain 2151 Rg-1. Lane 1, the 10% CH₃OH in CHCl₃ eluate from Florisil column chromatography of alkaline-stable lipid; lane 2, purified lipopeptide I (LP-I) from 2151 Rg-1; lane 3, purified lipopeptide II (LP-II) from 2151 Rg-1; and lane 4, purified ssGPL-2 from 2151 SmD. The thin layer plate was developed six times in CHCl₃-CH₃OH (12:1).
were assigned to the four amide groups of the tripeptide-amino alcohol core (Fig. 4A). The exact assignment of these protons to specific amino acids was accomplished by two-dimensional COSY 'H NMR (Fig. 4B). The coupling of the amide protons to their respective α-C protons was defined through their connectivity to the protons of the side groups (Table I).

'H NMR also revealed that the fatty acyl function of the lipopeptide was monounsaturated, as seen by the triplet at δ 5.32 ppm (Fig. 4). The two-dimensional COSY 'H NMR demonstrated the presence of a β-OH-fatty acid, with the resonance of the β-C proton apparent at δ 5.01 ppm. This proton was coupled to the α-C protons of the fatty acid which resonated at δ 2.46 ppm and also to the adjacent protons of the fatty acyl chain at δ 1.45 ppm (Fig. 4). Similar results were obtained with lipopeptide I; however, resolution was not as sharp because of unexplained degradation of the molecule during per-O-acetylation. Thus, GC and 'H NMR analyses of the lipopeptides clearly demonstrated the presence of the three amino acids (D-Phe, D-allo-Thr, D-Ala) and the one amino alcohol (L-alaninol), which constitute the peptide-amino alcohol core of the GPLs. Additionally, the 'H NMR and the two-dimensional COSY 'H NMR provided strong evidence for the presence of the very monounsaturated β-OH fatty acid that is characteristic of the GPLs of M. avium (26, 33).

Structures of Lipopeptides I and II—To confirm the sequence of the amino acids within the peptide core as well as the length and degree of unsaturation of the fatty acid, the native lipopeptides I and II were analyzed by FAB-MS (Fig. 5). The native lipopeptide II demonstrated a pseudomolecular ion ([M + H]⁺ of m/z 871 (Fig. 5B), suggesting that the tripeptide-amino alcohol core was linked to a C32:1-monounsaturated-β-OH fatty acid. The presence of such a fatty acid was consistent with the appearance of the α, m/z 596, b1, m/z 624, and b2, m/z 725 fragment ions. The a1 and b1 ions further demonstrated that the D-Phe was N-linked to the hydroxy C32:1 fatty acid, and the b2 ion confirmed that the next amino acyl function was D-allo-Thr. The y3+2 and x3 ions linked to the α-carbon of the Trp ring. This proposal was consistent with the appearance of the y3, m/z 248 and x3, m/z 173 ions that demonstrated the D-allo-Thr carboxyl terminus was linked to the Ala-alaninol terminus; the m/z 230 ion corresponds to the y3 fragment ion minus H2O (Fig. 5, A and B). Lipopeptide I yielded the same y3+2 and x3 ions as lipopeptide II (Fig. 5A); however, the a1, b1, and b2 fragment ions and the pseudomolecular ions were all 2 mass units lower, suggesting the presence of a C32:1-diunsaturated-β-OH fatty acid.

FAB-MS of the per-O-acetylated lipopeptides showed molecular ions of ([M + H]⁺ of m/z 995 for lipoprotein I and ([M + H]⁺ of m/z 997 for lipoprotein II, an increase in accord with the addition of three acetyl groups, as expected. The ensuing fragment ions (results not shown) served to confirm the conclusions already reached from FAB-MS analysis of the native, underivatized lipopeptides. The findings in total provided convincing evidence for the structures: C32:1-β-OH-fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-Alaninol for lipopeptide I and C32:1-β-OH-fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-Alaninol for lipopeptide II.

FAB-MS was also performed on the purified ssGPL-2 from the SmD variant to establish whether it also possessed the fatty acyl functions observed in the more primitive lipopeptides. Negative ion FAB-MS yielded a clear pseudomolecular ion ([M - H]⁻ of m/z 1,507, suggestive of the presence of a C32:1-diunsaturated-β-OH fatty acid (3-hydroxytricentand-1.20 αCH CH2 1.40 αCH 1.20 αCH

Table I

| Compound and group | ppm δ | Coupled to |
|--------------------|-------|------------|
| Alanine            | 4.25  | CH, NH     |
| Alanine            | 1.40  | αCH        |
| Phenylalanine      | 4.50  | CH, NH     |
| Phenylalanine      | 1.40  | αCH        |
| Allothreonine      | 4.45  | CH, NH     |
| Fatty acid         | 3.20  | αCH        |
| Fatty acid         | 2.95  | αCH        |
| CH2:CH=CH2         | 5.32  | CH, CH2, αCH |
| CH2:CH=CH2         | 2.00  | CH, CH2, αCH |
| CH3(OH)            | 5.01  | αCH, CH2, αCH |
| CH3(OH)            | 2.46  | αCH, CH2, αCH |
| CH2:CHOAc          | 1.45  | αCH, CH2, αCH |

*The multiplet centered at δ 7.26 ppm can be assigned by its chemical shift to the aromatic ring protons of Phe (34).

*Indicates the two CH2 groups of the fatty acid chain which are linked to the CH=CH.

*Indicates the CH2 groups of the fatty acid chain.

*Indicates the single CH2 group of the fatty acid chain linked to the β-carbon.

Fig. 4. 'H NMR of per-O-acetylated lipopeptide II. Panel A, one-dimensional 'H NMR. CHCl3 was assigned the chemical shift of δ 7.24. (Solvent was CDCl3.) Panel B, two-dimensional COSY 'H NMR.
FIG. 5. FAB-MS of purified lipopeptides I and II. Panel A, FAB-MS of lipopeptide I. The depicted structure of lipopeptide I has a molecular weight of 868. Panel B, FAB-MS of lipopeptide II. The depicted structure of lipopeptide II has a molecular weight of 870.

The fatty acid esters of lipopeptide II were obtained by methanalysis. GC-MS analysis of the (CH₃)₃Si derivative of the methyl esters confirmed that the major fatty acid of lipopeptide II was, indeed, a C₃₂₅ monounsaturated 3-OH fatty acid (Fig. 7). In addition, this type of analysis revealed that fatty acylation of the lipoprotein II is heterogenous in that C₂₀₋₂₄, C₂₄₋₂₈, and C₂₈₋₃₂ fatty acids were also detected in minor amounts. This heterogeneity was also observed in the FAB-MS of the lipopeptide II, in which ions 14 and 28 mass units higher than the (M + H)⁺ ion were observed (Fig. 5).

DISCUSSION

The isolation of spontaneous bacterial mutants on the basis of colony morphology has played a pivotal role in the elucidation of biosynthetic pathways for cell wall products and their implication as virulence factors. This point is best exemplified by the deep rough and semirough mutants of Salmonella spp. (35, 36), which allowed for the identification of genes and their enzymes responsible for lipopolysaccharide elaboration (35-39) and the definition of lipid A as the fundamental and toxic core of lipopolysaccharide (40, 41). Members of the M. avium complex are well known for their ability to express both rough and smooth colony forms (13, 14). However, in comparison with the Enterobacteriaceae, previous biochemical analyses of these morphological variants has not been as fruitful. Initial observations by Fregnan et al. (42) and Schaefer et al. (13) indicated that rough colony-forming variants of M. avium were devoid of ill defined surface structures. A subsequent study by Barrow and Brennan (15), employing rough and smooth variants of M. intracellulare serovar 20, firmly established that a consequence of rough colony formation was the absence of both the ns- and ssGPLs. Barrow (43) subsequently reported the presence of phenylalanine-containing lipopeptides in rough variants arising from cultures of M. avium serovars 4, 8, and 20. However, further analysis revealed the presence of phenylalanine, isoleucine, alanine and the apparent absence of threonine and alaninol, and thus these products appeared to be unrelated to the GPLs (48).

The present recognition of two nonglycosylated lipopep-
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The mechanism of the synthesis of such lipopeptides is unknown. Two separate means exist in prokaryotes for nonribosomal peptide biosynthesis, the principles of either of which may apply to the short tripeptide-amino alcohol core of the GPLs. The first possibility involves a form of direct synthesis in which the amino acids are added directly to an acceptor through the intervention of ATP. The muramyl-tetrapeptide unit of peptidoglycan is synthesized in this way (46). In the case of the peptide antibiotics, the amino acids are attached through thiol groups to a polyenzyome complex, and the peptide bond is subsequently formed through successive pantethenic aidened transpeptidation-transthiolation steps (47). If this latter mechanism were to apply to GPL biosynthesis, then it is likely that the final step in lipopeptide synthesis would involve transfer of the full peptide unit to the fatty acyl function. David et al. (17) demonstrated that the addition of m-fluoro-phenylalanine to cultures of M. avium inhibited GPL biosynthesis and, specifically, the incorporation of radioiodated amino acids into lipid. They also showed that D-cycloserine inhibited L-Ala racemization to D-Ala, resulting in a 20% inhibition of Ala incorporation into lipid without affecting the incorporation of allo-Thr or Phe. Even though these results do favor the direct synthesis route, additional lipopeptide intermediates of this pathway need to be isolated for definitive proof. From earlier work on the genes encoding the GPL antigens of M. avium (16), it was obvious that the ssGPLs are intermediates of the ssGPLs. However, further work is required to determine whether the oligosaccharide part of the ssGPLs is formed on a lipid carrier and then transferred to the nsGPL or whether the sugars are added singly and sequentially to the growing O-linked oligosaccharide chain of the GPLs.

Clearly, the isolation and characterization of a variety of "deep" rough mutants of M. avium are a major development in the goal of elucidating the biosynthetic pathway of the GPLs. They also provide the means to examine the roles of GPLs in the disease processes induced by M. avium, specifically in eliciting an immunosuppressive response (8).

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