The phenolic mycoside of *Mycobacterium ulcerans*: structure and taxonomic implications

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Mycobacterium ulcerans and some pathogenic mycobacterial species elaborate wax A consisting of related long-chain β -diol components (phthiocerol and related compounds) esterified by multimethyl-branched fatty acids. With the exception of *M. ulcerans*, wax A-containing mycobacteria also synthesize glycosylated phenol phthiocerol diester and related compounds: the so-called phenolic mycosides. In a deliberate effort to characterize this latter class of compounds in *M. ulcerans*, 20 strains were examined. Phenolic mycosides were found in two strains. Application of chemical analyses, including one- and two-dimensional NMR spectroscopy, allowed the structural elucidation of glycolipids identified as 3-*O*-methyl- α -L-rhamnosyl phenol phthiocerol diphthioceranate and related compounds which correspond to mycoside G, previously characterized in *M. marinum* by other investigators. As phenolic mycosides are highly species-specific molecules, this finding stresses the close phylogenetic link between *M. marinum* and *M. ulcerans*. Incidentally, a survey of the mycolate content of *M. ulcerans* showed that methoxymycolate could not be detected in three strains.

Introduction

Mycobacterium ulcerans is the aetiological agent of an indolent, necrotizing skin disease. A review (Portaels, 1989) has been devoted to the epidemiology of this infection which is endemic in some defined areas of the southern hemisphere (Hayman, 1984). Strains are characterized by slow growth occurring only at 30 °C and which may require several months even after subculture. Consequently, classical identification is difficult to perform and chemotaxonomic markers are of high value. *M. ulcerans* synthesizes α -, methoxy-, and ketomycolates, like several other mycobacterial species including tubercle bacilli, *M. marinum*, *M. kansasii* and *M. gastri* (Daffé *et al.*, 1983, 1991b; Minnikin *et al.*, 1984a). Moreover, *M. ulcerans*, *M. leprae* (Daffé & Lanéeelle, 1988) and the limited set of the above-mentioned species elaborate the so-called wax A composed of long chain β -diols (the phthiocerol family: Fig. 1, compounds I–III), with each mole of β -diol being esterified by two moles of multimethyl-branched fatty acids (Fig. 1, compound VII). An aromatic derivative, structurally related to phthiodiolone, called phenol phthiodiolone (Fig. 1, compound VI) has also been characterized in *M. ulcerans* (Daffé *et al.*, 1984). The distribution of all

these related lipids (phthiocerol, phthiodiolone, phenol phthiocerol and phenol phthiodiolone), as well as the nature of the fatty acids esterifying them, has been reviewed recently (Daffé & Lanéeelle, 1988). With the exception of *M. ulcerans*, glycosylated phenol phthiocerol diester and related compounds, called phenolic mycosides, have been found in species able to synthesize wax A (Daffé & Lanéeelle, 1988). Phenolic mycosides represent a class of mycobacterial antigens on which ELISA serodiagnostic tests rely (Brett *et al.*, 1983; Cho *et al.*, 1983; Martin-Casabona *et al.*, 1989; Torgal-Garcia *et al.*, 1988; Young & Buchanan, 1983).

However, wax A, as well as phenolic mycosides, are not present in all strains of a given species (Daffé & Lanéeelle, 1988; Daffé *et al.*, 1991a; Lévy-Frébault & Varnerot, 1989). In the present study we investigate the distribution of some specific lipids, including mycolic acids and phthiocerol derivatives, in order to ascertain chemotaxonomic markers helpful for the identification of *M. ulcerans* and to undertake a deliberate search for phenolic mycosides. We were able to detect the presence of large amounts of an apolar phenolic mycoside in two *M. ulcerans* strains and have elucidated the structure in this study.

Fig. 1. Formulae of mycobacterial phthiocerol, phenol phthiocerol and related compounds, and multimethyl-branched fatty acids. I-III, $m = 14-20$; IV-VI, $m = 16-22$; VII, $n = 12-20$ and $p = 3-4$.

Methods

Strains studied. Twenty *M. ulcerans* strains were included in the study (Table 1). Representatives of other mycobacterial species were also used: *M. marinum* CIPT 14012 0003, *M. avium* CIPT 14031 0002 (ATCC 25291), *M. gastri* CIPT 14034 0001 (ATCC 15754) and *M. bovis* BCG CIPT 14004 0001 (strain Pasteur).

Growth conditions. *M. ulcerans* strains were cultivated on Löwenstein-Jensen medium (Diagnostics Pasteur) or on a biphasic medium consisting of a Löwenstein-Jensen solid phase (75 cm² surface area) and 7H9 Middlebrook liquid phase (10 ml) (B. Carbonnelle, personal communication). Medium was distributed in plastic screw-capped flasks. After inoculation, flasks were incubated at 30 °C in an upright position and rolled once, once a day during the period of incubation. Serial subcultures performed in these conditions allowed us to obtain a substantial cell yield after a 3-4 week incubation period. *M. marinum* was grown on Sauton's medium (Sauton, 1912) at 33 °C for 4 weeks and its specific phenolic mycoside G was isolated in the same way as that of *M. ulcerans* (see below). Other strains used for controls for phenolic mycoside detection were cultivated on Löwenstein-Jensen medium.

Mycolic acid and other fatty acid analyses. Extraction, purification and derivatization as well as TLC were performed as previously described (Daffé *et al.*, 1983). Briefly, cells were scraped from

Löwenstein-Jensen medium and saponified in 5% (w/v) KOH in 2-methoxyethanol (methylcellosolve) containing 12% (v/v) H₂O, at 110 °C for 8 h to ensure complete hydrolysis of sterically hindered ester groups. After acidification with H₂SO₄, the solution was partitioned with diethyl ether. The ether phase was washed five times with distilled water and then dried. Fatty acids in extracts were esterified using a diethyl ether solution of diazomethane, freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluene sulphonamide (Diazogen; Janssen Chimica, Beerse, Belgium), for 15 min at room temperature. The solution was then dried under nitrogen and the lipids were redissolved in diethyl ether. TLC was performed in duplicate on K6 silica gel (Whatman). One plate was eluted with dichloromethane and the other plate was developed with a mixture of light petroleum ether (b.p. 50 °C) and diethyl ether (9:1, v/v) three times. GC was performed on fatty acid methyl esters using a Delsi 330 instrument equipped with a flame ionization detector and a $\frac{1}{8}$ in. $\times$ 3 m nickel column, filled with Chromosorb W coated with 5% OV-1. Nitrogen was used as the carrier gas with a flow rate of 30 ml min⁻¹. The temperature gradient of the column was programmed from 130 to 280 °C at a rate of 5 °C min⁻¹. Multimethyl-branched fatty acid and butyl glycoside derivatives were identified by GC using a Girdel chromatograph (model G30) equipped with a fused silica capillary column (25 m $\times$ 0.22 mm internal diam.) containing WCOT OV-1 (0.3 μ m film thickness, spiral, Dijon, France). A temperature gradient of 150-290 °C at 2 °C min⁻¹ was used.

Table 1. Strains of *M. ulcerans*

Strain number*	Source†	Mycolate type‡	Phthidiolone derivative§	Mycoside G
CIPT 109 0001 (ATCC 19423)	G. Buckle, Australia	I, III, IV	+	—
CIPT 109 0002	P. Laval, Mexico	I, IV	—	—
CIPT 109 0003 (HB 1673)	Dr Tarskis, Congo	I, III, IV	—	—
CIPT 109 0004	Dr Darasse	I, III, IV	+	—
CIPT 109 0005 (SP 151)	S. Pattyn, Zaire	I, III, IV	—	—
CIPT 109 0006 (SP 273)	S. Pattyn, Zaire	I, III, IV	+	—
CIPT 109 0007 (SP 286)	S. Pattyn, Zaire	I, III, IV	—	—
CIPT 109 0008 (SP 498)	S. Pattyn, Zaire	I, IV	—	—
CIPT 109 0009 (HB 3434)	M. Huet, Cameroon	I, III, IV	+	—
CIPT 109 0010 (HB 3435)	M. Huet, Cameroon	I, III, IV	+	—
CIPT 109 0011 (HB 3436)	M. Huet, Cameroon	I, IV	+	—
CIPT 109 0012 (HB 4145)	M. Huet, Cameroon	I, III, IV	+	—
CIPT 109 0013 (HB 4834)	M. Huet, Cameroon	I, III, IV	+	—
CIPT 109 0014 (HB 5091)	Dr Ménard, Cameroon	I, III, IV	+	—
CIPT 109 0015	J. Grosset, France	I, III, IV	—	—
CIPT 109 0016 (CIP 812023)	This laboratory	I, III, IV	+	—
CIPT 109 0018 (CIP 811479)	Guyana	I, III, IV	+	+
CIPT 109 0019 (ATCC 33728)	M. Tsukamura, Japan	I, III, IV	—	—
CIPT 109 0020 (CIP 841477)	Y. Pérol, France	I, III, IV	+	—
CIPT 109 0021 (CIP 890922)	B. Carbonelle, France	I, III, IV	—	+

* CIPT, Collection Institut Pasteur Tuberculose, Paris, France; ATCC, American Type Culture Collection, Rockville, MA, USA; HB, H. Boisvert (Paris, France); SP, S. Pattyn (Antwerp, Belgium); CIP, Collection Institut Pasteur, Paris, France.

† The donor of the strain is followed by the country of isolation. The country of isolation of strain CIPT 109 0004 is not known.

‡ Mycolate types I, III and IV refer to diunsaturated-, methoxy- and ketomycolates, respectively.

§ Phthidiolone and phenol phthidiolone diesters occur together. Some strains (+) accumulate phthiocerol derivatives in addition to the phthidiolone diester.

|| (+) indicates detection of a mycoside G-like glycolipid by TLC.

Extraction, detection and purification of the glycolipids. Bacteria were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) at room temperature for 1 week; lipid extracts were spotted onto HP-K high performance silica gel TLC plates (Whatman) and eluted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (9:1, v/v). Glycolipids were detected by spraying with 0.2% (w/v) anthrone in concentrated H_2SO_4 followed by heating at 110 °C.

For purification purposes, pooled extracts were dried, dissolved in CHCl_3 and applied to a Florisil (60/100 mesh) column (55 × 21 cm) and elutions were done with CHCl_3 and CHCl_3 containing 2, 5, 20 and 50% (v/v) CH_3OH . The collected fractions (50 ml) were dried and analysed by TLC. Final purification of the glycolipid was achieved by preparative TLC using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (95:5, v/v) as the developing solvent.

Miscellaneous analytical techniques. Alkaline hydrolysis of the glycolipid was performed as previously described (Daffé *et al.*, 1983).

Acid hydrolysis was performed with 2 M- CF_3COOH for 4 h at 110 °C; the sugars were demethylated (Bonner *et al.*, 1960) and the retention times on GC analysis of the trimethylsilyl derivatives of their (–)-2-butyl-glycosides were compared to that of (–)-2-butyl-L-rhamnoside and (–)-2-butyl-D-rhamnoside (Gerwig *et al.*, 1978).

NMR spectra were obtained in CDCl_3 with a Bruker AM 300 WB instrument at 25 °C. The two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment was conducted as described elsewhere (Daffé & Servin, 1989).

IR spectra were recorded on a Perkin-Elmer instrument model 1600 series FTIR.

Optical rotation was determined in CHCl_3 solution (dried over CaCl_2) with a Perkin-Elmer spectropolarimeter model 241 in a temperature controlled cell (20 °C).

Results

Mycolic acid and fatty acid derivative profiles

Previous studies, using only a few strains, showed that *M. ulcerans* synthesizes three types of mycolates, namely diunsaturated- (type I), methoxy- (type III) and ketomycolates (type IV) (Daffé *et al.*, 1983, 1991b). In order to ascertain the chemotaxonomic significance of this mycolic acid pattern, 20 strains were analysed for their mycolate content. As shown in Table 1, all the strains did not present the same mycolic acid profile. Strains CIPT 109 0002, 109 0008 and 109 0011 did not accumulate methoxymycolate in sufficient quantity to be detected on TLC plates (Table 1). This feature was not related to the geographical source, as these strains were from Mexico, Zaire and Cameroon, respectively. Fatty acid methyl esters were then examined by GC for the presence of specific chemotaxonomic markers of *M. ulcerans*. The comparative study revealed, in all strains, the presence of a high content of the characteristic C_{27} phthioceranate methyl ester which represented up to 40% of the total fatty acid derivatives detectable, a specific feature of *M. ulcerans* (Daffé *et al.*, 1984).

Fig. 2. ^1H -NMR spectrum of the phenolic mycoside of *M. ulcerans* (300 MHz, CDCl_3). The labelled resonances correspond to those of protons marked in Fig. 3 except for resonances c ($\text{CH}_3\text{—CH}_2\text{—CO—}$ in phenol phthiodiolone), f ($\text{CH}_3\text{O—CH—CH}_3$ in phenol phthiocerol B) and o (CHCl_3).

Detection and preliminary characterization of the phenolic mycoside

In the crude lipid extracts of two strains of *M. ulcerans* (CIPT 109 0018 and 109 0021), a major glycolipid was detected by TLC. It shared the same chromatographic properties with the two monoglycosyl phenol phthiocerol diesters, namely phenolic mycosides B and G isolated from *M. bovis* and *M. marinum*, respectively, but was clearly different from multiglycosylated phenolic mycosides (Daffé *et al.*, 1987). The optical rotation of the purified glycolipid was found to be -16° (CHCl_3 , $c=0.9$), a value close to those previously found for mycosides B and G (Daffé *et al.*, 1987). The IR spectrum of the glycolipid was identical to those previously published for phenolic mycosides A, B and G (Gastambide-Odier & Sarda, 1970; McLennan *et al.*, 1961; Navalkar *et al.*, 1965) and indicated the occurrence of a phenolic group (absorption bands at 1615, 1535 and 825 cm^{-1}).

Carbohydrate constituent of the glycolipid

The presence in the ^1H -NMR spectrum (Fig. 2) of the glycolipid isolated from *M. ulcerans* of only one signal (m) assignable to the resonance of an anomeric proton ($\delta\ 5.52\text{ p.p.m.}$, 1H) indicated a monoglycosyl structure. In addition to the singlet due to the presence of the methoxyl group of phenolphthiocerol A (compound IV, Fig. 1) at $\delta\ 3.32\text{ p.p.m.}$ (signal g, Fig. 2), a second singlet was observed at $\delta\ 3.54\text{ p.p.m.}$ (signal h) assignable to the resonance of a methoxyl group located on the sugar

Table 2. Analysis of ^1H -NMR data for native and peracetylated derivatives of the monoglycosyl phenol phthiocerol diester of *M. ulcerans* and *M. marinum* (mycoside G)

Sugar ring proton	Chemical shift, δ (p.p.m.)		Coupling constant (Hz)
	Native	Peracetylated	
H-1	5.52	5.42	$J_{1,2} = 1.8$
H-2	4.25	5.49	$J_{2,3} = 3.9$
H-3	3.60	3.80	$J_{3,4} = 10$
H-4	3.57	5.04	$J_{4,5} = 10$
H-5	3.82	3.95	$J_{5,6} = 6.3$
H-6	1.38	1.17	—

residue. When the resonances of the different sugar protons were assigned (Table 2) it was easy to identify the glycosyl unit as rhamnosyl because of the coupling constant values (Haverkamp *et al.*, 1975). The configuration of the rhamnosyl unit was determined by NOESY which revealed an unambiguous space connectivity between the H-1 and H-2 resonances but not between those of H-1 and H-3 or H-5 (data not shown), establishing an α configuration (Yu *et al.*, 1986). The anomeric proton also showed a space connectivity with the phenyl protons. The location of the methoxyl group was deduced from the same experiment showing a connectivity between the resonance of H-2 and that of the methoxyl protons at $\delta\ 3.54\text{ p.p.m.}$ (data not shown), thus locating the methoxyl group at position 3 of the sugar residue. These data were confirmed by comparing the spectrum shown in Fig. 2 with those of mycosides B and G (2-O- and 3-O-methyl rhamnosyl phenol phthiocerol diesters, respectively). The spectrum of mycoside G, but not of mycoside B (Daffé *et al.*, 1988), was found to be superimposable on that shown in Fig. 2. The ^{13}C spectrum of mycoside G allowed the assignment of an α configuration to the rhamnosyl unit [$J_{\text{C-H}} = 168\text{ Hz}$ (Kasai *et al.*, 1979)] and confirmed the location of the methoxyl group at position 3 ($\delta\ 81\text{ p.p.m.}$; Haverkamp *et al.*, 1975). Analysis of the ^1H -NMR of the peracetylated glycolipid (Table 2) also confirmed the α configuration of the rhamnosyl unit: $\delta_{\text{H-5}} > 4.7\text{ p.p.m.}$, $\delta_{\text{H-6}} < 1.2\text{ p.p.m.}$, (Laffite *et al.*, 1978) and the location of the methoxyl group at position 3 (not shifted). The absolute configuration of the rhamnosyl units was determined after demethylation followed by butanolysis of the sugar constituent of both glycolipids isolated from *M. ulcerans* and *M. marinum* (mycoside G). L-Rhamnoside was identified in both glycolipid derivatives as the sugar co-chromatographed with derivatives of L-rhamnose but not D-rhamnose on GC. This finding is in agreement with the previous results on mycoside G (Villé & Gastambide-Odier, 1970).

Fig. 3. Proposed structure for the major constituent of the phenolic mycoside of *M. ulcerans*: 3-O-methyl- α -L-rhamnosyl phenol phthiocerol diester. The resonances of protons labelled a–n are shown in Fig. 2.

Aglycone moiety of the glycolipid

The structure of the phenol phthiodiolone of *M. ulcerans* has been established previously (Daffé *et al.*, 1984). Analysis of the ^1H -NMR spectrum confirmed the presence of an aromatic group (δ 6.98–7.10 p.p.m., signal n, Fig. 2) and of multimethyl-branched fatty acyl substituents (δ 1.14 p.p.m., signal d, Fig. 2) esterifying a β -glycol (δ 4.89 p.p.m., signal l, Fig. 2). The chemical shift observed for the latter signal was significantly downfield from that (δ 4.83 p.p.m.) recorded for other phenolic mycosides isolated from *M. tuberculosis* and *M. bovis* (Daffé *et al.*, 1987, 1988) but was similar to that of mycoside G (Dobson *et al.*, 1990). This effect is related to the *erythro* configuration in the β -glycol of wax A and phenolic compounds of *M. marinum* and *M. ulcerans* as compared to the *threo* configuration found in those of *M. tuberculosis* and *M. bovis* (Besra *et al.*, 1989, 1990).

The presence of a singlet assignable to a methoxyl group at δ 3.32 p.p.m. (1.5H, signal g, Fig. 2) indicated the presence of phenol phthiocerol A (compound IV, Fig. 1). Another singlet at δ 3.29 p.p.m. (0.5H, signal f, Fig. 2) was assigned to the resonance of the methoxyl group of phenol phthiocerol B (compound V, Fig. 1). The intensity of the resonances (signals g + f = 2H) due to the two phenol phthiocerol derivatives suggested that two-thirds of the native mycoside contained phenol phthiocerol moieties. Thus, it appeared likely that the remaining one-third of the native mycoside would contain phenol phthiodiolone diester as its aglycone moiety. The presence of a triplet at δ 1.05 p.p.m. (signal c, Fig. 2) attributable to the terminal methyl group of the phenol phthiodiolone derivative (Daffé & Servin, 1989; Dobson *et al.*, 1990; Minnikin & Polgar, 1967) confirmed the above hypothesis. The aglycone moiety appeared then to consist of phenol phthiocerols A, B and phenol phthiodiolone derivatives in a ratio 3:1:2 respectively.

By co-chromatography, using a capillary column and authentic standards, the fatty acyl substituents of the glycolipids of *M. ulcerans* and *M. marinum* were found to

be mainly 2,4,6-trimethyl tetracosanoyl; small amounts of lower and higher homologues were also present. These fatty acyl derivatives were previously demonstrated to belong to the phthioceranic series in both organisms (Daffé, 1991; Daffé & Lanéelle, 1988; Daffé *et al.*, 1984) in contrast to those found in wax A and phenolic mycosides of *M. tuberculosis* and other wax A-containing mycobacterial species (Daffé & Lanéelle, 1988).

Based on the data presented in this paper, the structure shown in Fig. 3 can be proposed for the major component of the phenolic mycoside of *M. ulcerans*. It corresponds also to that of mycoside G of *M. marinum*, (Dobson *et al.*, 1990; Villé & Gastambide-Odier, 1970).

Discussion

As the classical identification of *M. ulcerans* is hampered by the very slow growth of the organisms, we looked for specific chemotaxonomic markers: mycolates, multi-methyl-branched fatty acids and their natural forms (wax A and phenolic mycosides).

Previous studies showed that mycolates are a highly reproducible feature within a species (Daffé *et al.*, 1983; Lévy-Frébault *et al.*, 1983, 1986; Luquin *et al.*, 1991; Minnikin *et al.*, 1984a). Previously, when a mycolate profile has differed from that of the type strain, it has usually indicated a misidentification or a mixed culture (Lévy-Frébault *et al.*, 1986, 1987). The unique exception described so far is the case of some of the *M. bovis* BCG strains. It has been shown that Pasteur, Glaxo, Prague, Danish and Chinese BCG strains do not contain methoxymycolates (Daffé *et al.*, 1983; Minnikin *et al.*, 1984b). Strains of *M. ulcerans* also exhibit variability in methoxymycolate content, as demonstrated in the present study. A possible explanation of this may reside in the postulated biosynthetic relationship between keto- and methoxymycolates, the latter being derived from the former (Minnikin, 1982).

The fatty acids esterifying wax A and phenolic mycosides can be easily detected by GC and may consequently represent a reliable chemotaxonomic marker for identification of *M. ulcerans*. These fatty acids were previously demonstrated to belong to the phthioceranic series like those from *M. marinum* in contrast to those found in all other wax A-containing mycobacterial species which belong to the mycocerosic series (Daffé & Lanéeelle, 1988), the data stressing an obvious analogy between the fatty acid biosynthesis of *M. ulcerans* and *M. marinum*.

The present study clearly demonstrates that *M. ulcerans* may elaborate phenolic mycosides, the structures of which correspond to those of mycoside G of *M. marinum* (Dobson *et al.*, 1990; Villé & Gastambide-Odier, 1970). Only two of the *M. ulcerans* strains, out of the 20 included in the study, revealed the presence of mycoside G. This observation confirms the earlier finding showing that phenolic glycolipids are not reliable markers as they were not found in all the strains of a given species (Daffé & Lanéeelle, 1988). For instance, although the presence of specific phenolic mycosides of *M. bovis* and *M. tuberculosis* was typical for field isolates (Chatterjee *et al.*, 1989; Daffé *et al.*, 1989), the type strain of *M. bovis* does not reveal any mycoside B (Daffé & Lanéeelle, 1988); similarly some *M. tuberculosis* strains do not accumulate detectable amounts of PGL-Tb1 (Daffé & Lanéeelle, 1988; Daffé *et al.*, 1991a). Nevertheless, the presence of identical phenolic mycosides in different species may indicate close phylogenetic relatedness. For example, the two species *M. tuberculosis* and *M. bovis* are both able to synthesize identical mono- and diglycosyl phenolic mycosides (Daffé *et al.*, 1988, 1991a; Chatterjee *et al.*, 1989; Vercellone & Puzo, 1989) and DNA/DNA hybridization studies demonstrated that these two taxa belong to a unique genomic group (Baess, 1979). Similarly, *M. kansasii* and *M. gastri* accumulate the same phenolic mycosides (Gilleron *et al.*, 1990; Papa *et al.*, 1987; Vercellone *et al.*, 1988) and were found to be indistinguishable according to their rRNA sequences (Rogall *et al.*, 1990) and their fatty acid content (Daffé *et al.*, 1983). Classical identification methods, however, easily differentiate *M. kansasii*, a potential pathogen frequently isolated in clinical samples, from *M. gastri*, a non-pathogenic species (Wayne & Kubica, 1986). Consequently, although phenolic mycosides may not always be expressed, when they are, they may show a phylogenetic relationship in close agreement with genetic studies for taxa differentiated by classical identification methods and with distinct pathogenic behaviour. *M. marinum*, a rapidly-growing photochromogenic species, and *M. ulcerans*, a slowly-growing non-chromogenic species, are easily differentiated by cultural and biochemical tests as well by clinical presentation of the lesions they induce.

M. marinum gives cutaneous granuloma, usually self-limiting and *M. ulcerans* produces invasive skin ulcers. Nevertheless, the following characteristics are shared by *M. marinum* and *M. ulcerans*: (i) identical types and subtypes of mycolic acids (Daffé *et al.*, 1983, 1991b); (ii) the stereochemistry of both the methyl-branched- and the β -glycol chiral centres occurring in wax A and related phenolic analogues, opposite to those of the other mycobacterial species (Besra *et al.*, 1989, 1990; Daffé, 1991; Daffé & Lanéeelle, 1988; Daffé *et al.*, 1984); and (iii) the identical structure of their phenolic glycolipid. These features stress the close relationship between the two species. In support of this concept is the high genomic relatedness (86%) by spectrophotometric assay in DNA/DNA hybridization studies (Imaeda *et al.*, 1988). Further genomic studies will be required to establish the phylogenetic relationship between the different variants of *M. ulcerans* on the one hand and the *M. marinum* strains on the other.

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