Structural studies of cord factors from *Mycobacterium simiae* related to the capacity for tumour necrosis factor alpha (α -TNF) induction

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The structure of cord factor was studied in several strains of *Mycobacterium simiae*, including 'habana' TMC 5135, considered as highly immunogenic in experimental tuberculosis and leprosy. The mycolic acids liberated from cord factor were identified in all cases as α' -, α - and keto-mycolates. According to the general NMR and MS data, α' -mycolates were mono-unsaturated and contained from 64 to 68 carbon atoms, whereas α -mycolates mainly presented two 2,3-disubstituted cyclopropane rings and a chain length of 80–91 carbon atoms; keto-mycolates mostly contained one cyclopropane ring and 85–91 carbon atoms. Taking into account the ¹H-NMR results, strains varied in the ratio of the different mycolates, and the high levels of keto-mycolates found in the cord factors of TMC 5135 and ATCC 25275^T stood out. Notably, MS revealed that the odd carbon number series of α -mycolates (C87–C89) predominated in the cord factor of TMC 5135, in contrast to the remaining studied strains, in which the even (C84–C86) and odd carbon number series appeared more equal. The fine structural differences detected among the cord factors studied did not seem to be relevant to the general capacity of these molecules to induce the secretion of tumour necrosis factor alpha, as the cord factors from several strains of *M. simiae* (TMC 5135, IPK-342 and ATCC 25275^T) induced similar amounts of this cytokine in RAW 264.7 cells.

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INTRODUCTION

Mycobacterium includes the causative agents of tuberculosis and leprosy, and other saprophytic and opportunistic species, such as *Mycobacterium simiae* (Falkinham, 1996). The 'habana' group, integrated in this species, was originally isolated in Cuba from patients with pulmonary infections (Valdivia, 1973) and was revealed to be identical to *M. simiae* serotype I. Although some differences have been noted between 'habana' and other strains of *M. simiae* (Khoo *et al.*, 1996; Mederos *et al.*, 1998, 2007, 2008), the synonymy was clearly established some decades ago (Weiszfeiler & Karczag, 1976). Interest in the 'habana' group still remains, mainly because some researchers from

India demonstrated that 'habana' TMC 5135 presents interesting immunogenic properties in experimental tuberculosis and leprosy (reviewed by Mederos *et al.*, 2006). Moreover, Mederos *et al.* (2007) have reported that 'habana' TMC 5135 differs from other 'habana' strains and from *M. simiae* ATCC 25275^T in α -mycolic acid composition.

Mycolic acids are 2-alkyl-branched, 3-hydroxy long-chain fatty acids (Fig. 1) present in mycobacteria and related micro-organisms. In *Mycobacterium*, these compounds vary from 60 to 90 carbon atoms, being mainly linked to the arabinogalactan of the cell wall (McNeil *et al.*, 1991). However, they also appear in the cell envelope attached to trehalose, forming the structure of a glycolipid called cord factor (Brennan & Nikaido, 1995; Daffé & Draper, 1998). Mycolic acids from *Mycobacterium* can either contain additional oxygenated functions (as in keto- and methoxymycolates) or not (α - and α' -mycolates), and also show variations related to the presence of *cis* or *trans* di-

Abbreviations: ESI-IT-MS, electrospray ionization-ion trap-MS; ¹H-¹³C-HMBC, ¹H-¹³C heteronuclear multiple bond coherence spectroscopy; ¹H-¹³C-HMQC, ¹H-¹³C heteronuclear multiple quantum coherence spectroscopy; ¹H-¹H-COSY, ¹H-¹H homonuclear correlated spectroscopy; IL, interleukin; α -TNF, tumour necrosis factor alpha.

Fig. 1. General structure of major α - (a, b) and keto-mycolates (c) of *M. simiae* (Mederos *et al.*, 2007) employed to assign the resonances of ^1H -NMR (Fig. 2) and ^{13}C -NMR (Table 2) spectra. α -Mycolates present two 2,3-disubstituted cyclopropane rings, one *cis* and one *trans* (a) or two *cis* (b); keto-mycolates mainly have one *trans*-2,3-disubstituted cyclopropane ring (c). Minor α - and keto-mycolates containing one *trans* double bond instead of one cyclopropane ring are also present in *M. simiae* (not shown) (Mederos *et al.*, 2007). The structure of α' -mycolates (C64–C68) is indicated in (d); these compounds only contain one *cis* double bond ($-\text{CH}=\text{CH}-$, Fig. 2). Carbon atoms at positions 2 and 3 of the mycolic acids are indicated in (a); note that C-2 and C-3 are two chiral centres of the molecule with configuration *R/R* in naturally occurring mycolic acids.

substituted cyclopropane rings and unsaturations (Minnikin, 1982; Watanabe *et al.*, 2001).

Cord factor, first detected in *Mycobacterium tuberculosis* as a toxic substance (Bloch, 1950) and identified as 6,6'-dimycoloyl trehalose (see Fig. 2) (Noll *et al.*, 1956), is considered to play an important role in the pathogenesis of tuberculosis (reviewed by Hunter *et al.*, 2006, 2009). Several experimental results have indicated that cord factor is the major molecule secreted by mycobacteria inside macrophages (Rhoades *et al.*, 2003), and that its mycoloyl substituents can influence some aspects of the virulence of *M. tuberculosis* by modulating the production of some cytokines (Dao *et al.*, 2008; Rao *et al.*, 2005, 2006). Notably, mutants of *M. tuberculosis* altered in the capacity for synthesis of cyclopropane rings show alterations in virulence (Riley, 2006). More precisely, the lack of synthesis of *trans*-cyclopropane rings gives rise to a hypervirulence phenotype (Rao *et al.*, 2006). Mycolic acids from the delipidated strain 'habana' TMC 5135 contain high amounts of *trans*-cyclopropane rings (Mederos *et al.*, 2007), leading to the idea that this fact could be reflected in extractable mycolic acid-containing lipids, such as cord factor, and could influence the immunogenicity of the strain. The present work determines the structure of the cord factor of *M. simiae* and reveals some particular characteristics in strain 'habana' TMC 5135, although its capacity to induce tumour necrosis factor alpha (α -TNF) was, in general, similar to that of other strains of the species.

METHODS

Strains studied. Five strains of *M. simiae* were included in the present study: TMC 5135, ATCC 25275^T, IPK-220, IPK-337 and IPK-342. All of them belonged to the collection of the IPK (Instituto de Medicina Tropical Pedro Kouri), La Habana, Cuba. They were

cultivated in liquid UIT-L medium (Mederos *et al.*, 1992) at 35 °C for 3 weeks, harvested, washed with saline, killed at 120 °C for 20 min and dried in a vacuum over P_2O_5 (Mederos *et al.*, 2007).

Extraction and purification of cord factor. Cells (2–3 g) were extracted with chloroform/methanol (1:1, v/v) and chloroform/methanol (2:1, v/v) (twice) overnight at room temperature with agitation (100 r.p.m.). The extracts were collected and mixed, evaporated to dryness and washed with chloroform/methanol/water (8:4:3, by vol.). Lipids in the lower phase were collected, evaporated to dryness and precipitated (three times) in methanol (–30 °C, overnight). The resulting insoluble extract (containing cord factor and other lipids) was subjected to silica gel liquid chromatography (particle size 0.040–0.063 mm, Merck), employing methanol in chloroform (0, 2, 5, 10, 30 and 50%) as solvent. Fractions were collected and analysed by TLC (silica gel plates, Merck), employing chloroform/methanol (9:1, v/v) as solvent. Cord factor was recovered in the 10% methanol fraction and repurified by preparative TLC (silica gel plates, Merck). In this case, and due to the presence of small amounts of contaminants (apolar glycopeptidolipids), the plates were developed with acetone (three times) followed by chloroform/methanol (9:1, v/v) (once). The purity of cord factor was checked by TLC, as above.

Structural analysis of cord factor. ^1H -NMR and ^1H - ^1H homonuclear correlated spectroscopy (^1H - ^1H -COSY) analyses of cord factor, dissolved ($\sim 4 \text{ mg ml}^{-1}$) in deuteriochloroform/deuteriomethanol (5:1, v/v), were carried out in a Bruker instrument (600 MHz) at 298 K. Given the differences initially noted for the chemical shifts of H-5/H-5' and H-6b/H-6'b among the cord factors of some strains (see Results), and to check for a possible solvent effect, ^1H -NMR analyses were also done under the same conditions at various ratios (v/v) of deuteriochloroform/deuteriomethanol: 30:1, 10:1 and 5:1, employing cord factor of TMC 5135. This compound, dissolved in deuteropyridine, was also studied by ^1H -NMR and ^1H - ^1H -COSY, because an influence on chemical shifts related to variations in the configuration of the chiral centres at C-2 and C-3 of mycolates (see Fig. 1) has been noted (Nishizawa *et al.*, 2007). ^{13}C -NMR (150 MHz), ^1H - ^{13}C heteronuclear multiple quantum coherence spectroscopy (^1H - ^{13}C -HMQC) and ^1H - ^{13}C heteronuclear multiple bond coherence spectroscopy (^1H - ^{13}C -HMBC) experiments were performed with cord factor of TMC 5135 in the above NMR instrument. Both ^1H - ^{13}C -HMQC and

Fig. 2. ^1H -NMR spectrum (600 MHz in deuteriochloroform/deuteromethanol, 5:1, v/v) of cord factor from *M. simiae* ATCC 25275^T. The general structure of cord factor is included, indicating the protons (H-1 to H-6 and H-1' to H-6') and carbons (1 to 6 and 1' to 6'). *cis*-cyclo, *cis* di-substituted cyclopropane ring; *trans*-cyclo, *trans* di-substituted cyclopropane ring. t, terminal; b, branched; MeOD, deuteromethanol; *cis*-CH=CH- (α' -mycolates) and *trans*-CH=CH- (α - and keto-mycolates) are also indicated (see Fig. 1). In some cases the signals correspond to the underlined protons.

^1H - ^{13}C -HMBC served to establish the connectivities between ^1H and ^{13}C and, hence, identify the carbons of trehalose and mycoloyl residues of cord factor.

To estimate the relative amounts of the different mycolates, as well as the *trans*-/*cis*-cyclopropane ratio, the ^1H -NMR spectra were employed, applying, with slight modifications, some of the formulae proposed by Watanabe *et al.* (2001). The integrals of the signals at 5.27 p.p.m. (*cis* double bond: -CH=CH-, 2H, integral/2) (see Results, Fig. 2) and 2.45 p.p.m. (methyne adjacent to a -CO-, 1H) (see Results, Fig. 2) were evaluated for the contribution of, respectively, α' - and keto-mycolates. The contribution of α -mycolates was calculated from the formula:

$$8\alpha + 4\text{keto} = (\text{cyclopropane rings}) + (\text{trans double bond}) + (-\text{CH}_2\text{-adjacent to the trans double bond})$$

(Note that there are eight and four cyclopropane protons, respectively, for α - and keto-mycolates, and that low amounts of both mycolates contain *trans* double bonds instead of cyclopropane rings; see Fig. 1.) It was taken into account that the broad signal at 0.58 p.p.m. (3H) (see Results, Fig. 2) includes one proton that does not belong to the *cis*-cyclopropane ring. The *trans*-/*cis*-cyclopropane ratio was deduced from the integrals of the corresponding regions of the spectrum (see Results, Fig. 2). The *trans* double bonds/cyclopropane rings ratio was calculated in a similar way.

A small amount of cord factor (1 mg) was saponified overnight at 90 °C in 15% (w/v) KOH in methanol, and the liberated mycolic acids were extracted with hexane after acidification of the mixture (20% H_2SO_4) at pH 2. The extract of mycolic acids was washed with distilled water to pH 6–7, dried with anhydrous Na_2SO_4 , dissolved in chloroform/methanol (2:1, v/v) and analysed by electrospray ionization-ion trap-MS (ESI-IT-MS) in negative mode in an MSD-IonTrap-VL (Agilent) instrument, with direct infusion of the sample (0.6 ml h^{-1}). The electrospray needle was set at 3.5 kV and compressed N_2 employed as the nebulization gas (69 kPa, 325 °C). The voltage applied was 80 V. Spectra were recorded over the mass range m/z 100–2000 at 3–5 s. Monoisotopic masses (obtained from the empiric formulae at ChemCalc; <http://www.chemcalc.org>) and previous data (Mederos *et al.*, 2007) served as a reference for identification of mycolic acids. For comparative purposes, mycolic acids from delipidated cells (50 mg) were also studied in a similar way. Both cord factor and delipidated cells from TMC 5135 were analysed in duplicate (independent analyses).

α -TNF response of RAW 264.7 cells to cord factor. The method described by Rao *et al.* (2006) was followed with some modifications. Cord factor was resuspended by sonication in 2-propanol (Merck) (1 mg ml^{-1}) and layered [0 μg (2-propanol alone), 0.1, 1 and 10 μg] in 24-well tissue culture plates (Nunc). RAW 264.7 cells (mouse

Fig. 3. ESI-IT-MS spectrum of mycolic acids from cord factor of *M. simiae* 'habana' TMC 5135. The different structural types (α' -, α - and keto-mycolates) are indicated, together with the chain length. The m/z values correspond to the $(M-H)^{-1}$ ions (see Table 4). ?, Contaminant from adsorbant.

leukaemia macrophage cell line, purchased from ATCC) were added (10^6 cells) in 500 μ l Dulbecco's Modified Eagle's Medium (DMEM) (high glucose), supplemented with 10% fetal calf serum, penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$), and incubated at 37°C in 5% CO_2 for 24 h. All cultures were negative for mycoplasma and LPS. Supernatants were collected, centrifuged and kept at -30°C until analysis. The amount of α -TNF was analysed using the BD OptEIA mouse α -TNF kit (BD Bioscience), following the recommendations of the manufacturer. The analyses were done in duplicate experiments (and duplicated assays), and general statistical differences (significance at $P < 0.05$) were estimated by two-way analyses of variance (SPSS 15.0) and the least significant difference test.

RESULTS

Structure of cord factor

Cord factor from *M. simiae* strains was unambiguously identified as 6,6'-dimycoloyl trehalose by ^1H -NMR, ^1H - ^1H -COSY-NMR, ^{13}C -NMR, ^1H - ^{13}C -HMQC and ^1H - ^{13}C -HMBC. The ^1H -NMR spectra obtained (Fig. 2) were in general agreement with those reported in the literature for natural and synthetic cord factors (Niescher *et al.*, 2006; Nishizawa *et al.*, 2007). The anomeric protons of trehalose (H-1/H-1') (Fig. 2) resonated at 4.98 p.p.m., and $J_{1,2}$ was 3.9 Hz, demonstrating an α, α' configuration (Lin *et al.*, 2007). Moreover, two of the H-6 (H-6a/H-6'a) appeared at 4.50 p.p.m. and the two remaining (H-6b/H-6'b) at 4.08 p.p.m., indicating that the C-6 hydroxyl groups (C-6/C-6') were esterified (Lin *et al.*, 2007). Unexpectedly, the

signals of H-5/H-5' and H-6b/H-6'b of trehalose from cord factor of the strains 'habana' TMC 5135 and IPK-220 overlapped at 4.05 p.p.m. (results not shown), although this fact was attributed to a solvent effect (see below). H-2/H-2', H-3/H-3', H-4/H-4' and H-5/H-5' (Fig. 2) resonated at, respectively, 3.42, 3.67, 3.22 and 4.02 p.p.m. The spectrum of strain IPK-220 showed reverse chemical shifts for H-5/H5' and H-6b/H-6'b (results not shown). On the other hand, H-3/H-3' from cord factor of TMC 5135 overlapped with the signal of deuteromethanol (results not shown).

To check the apparent anomalies noted in the ^1H -NMR spectra of TMC 5135 and IPK-220, cord factor of the first strain was reanalysed employing the following ratios (v/v) of deuteriochloroform/deuteromethanol: 30:1, 10:1 and 5:1. The results are presented in Table 1, and prove that the chemical shifts of H-5/H5' and H-6/H6' were the most influenced; thus, the overlap mentioned above was attributed to a solvent effect and no anomalies were actually considered to be present in the spectra. Moreover, when using deuteropyridine as solvent, the ^1H -NMR spectrum of cord factor from TMC 5135 (not shown) (H-1/H1', 5.63 p.p.m.; H-2/H-2', 4.00 p.p.m.; H-3/H-3', 4.46 p.p.m.; H-4/H-4', 3.92 p.p.m.; H-5/H5' and H-6a/H6'a, 4.92 p.p.m.; H-6b/H-6'b, 4.46 p.p.m.) did not differ from those reported by Nishizawa *et al.* (2007) for compounds presenting a 2*R*,3*R*/2'*R*,3'*R* configuration. On the other hand, the ^{13}C -NMR spectrum of cord factor from TMC 5135 (Table 2) was similar to those of other cord factors reported in the literature (Nishizawa *et al.*, 2007).

Table 1. Chemical shifts (p.p.m.) (^1H -NMR) of the trehalose protons of cord factor (see Fig. 2) from *M. simiae* ('habana') TMC 5135 at various deuteriochloroform/deuteromethanol ratios

-CH=CH- was included as a reference.

Proton	Deuteriochloroform/deuteromethanol ratio (v/v)			
	30:1	10:1	5:1*	5:1†
H-1/H-1'	4.92	4.95	4.96	4.98
H-2/H-2'	3.47	3.43	3.42	3.42
H-3/H-3'	3.71	3.69	3.68	‡
H-4/H-4'	3.14	3.18	3.20	3.22
H-5/H-5'	4.29	4.16	4.08	4.05
H-6a/H-6'a	4.73	4.60	4.52	4.50
H-6b/H-6'b	3.80	3.94	4.01	4.05
-CH=CH-	5.27	5.27	5.27	5.27

*Second analysis.

†First analysis.

‡Overlaps with deuteromethanol.

Acyl substituents were identified as α' -mycoloyl (signal at 5.27 p.p.m., due to a *cis* double bond, -CH=CH-), α -mycoloyl (signals at -0.40, 0.49 and 0.58 p.p.m., due to a *cis* di-substituted cyclopropane ring) and keto-mycoloyl (signals at 0.98 and 2.45 p.p.m., due to, respectively, a methyl branch and a methyne adjacent to the -C=O)

Table 2. ^{13}C -NMR analysis of cord factor from *M. simiae* ('habana') TMC 5135 (see Figs 1 and 2 for reference)

Chemical shifts are given in p.p.m. (in some cases they correspond to the underlined carbons). The different carbons were attributed by means of ^1H - ^{13}C -HMQC and ^1H - ^{13}C -HMBC experiments.

^{13}C	Chemical shift
Trehalose	
C1/C1'	94.44
C2/C2'	71.33
C3/C3'	72.75
C4/C4'	70.92
C5/C5'	69.88
C6/C6'	63.87
Mycoloyl substituents	
-CO- (keto-mycolates), - ^1COO -	175.35
-CH=CH- (<i>cis</i>) α' -mycolates	129.76
^3C	72.46
^2C	52.43
<i>trans</i> -Cyclopropane	10.32, 19.50, 25.19
<i>cis</i> -Cyclopropane	10.72, 15.64
-CH ₃	13.87
<u>CH</u> ₃ -CH-CO-	16.12
CH ₃ - <u>CH</u> -CO-	46.23
- <u>CH</u> ₂ -CO-	41.08
CH ₃ -CH- <i>trans</i> -cyclopropane	37.29
<u>CH</u> ₃ -CH- <i>trans</i> -cyclopropane	18.49
-CH ₂ -	22.53–34.69

(Fig. 2) (Mederos *et al.*, 2007; Watanabe *et al.*, 1999). Other signals related to a *trans* di-substituted cyclopropane ring (0.04–0.07 p.p.m.) (due to α - and keto-mycoloyl substituents), -CH₂- and -CH₃ (terminal and branched) were also present in the spectra (Fig. 2), as were those related to the presence of *trans* double bonds (doublet at 0.87 p.p.m., CH₃-CH-CH=CH-; and multiplets at 5.17 and 5.23 p.p.m., *trans*-CH=CH-) (Fig. 2) (Mederos *et al.*, 2007; Watanabe *et al.*, 1999). Details of the ^{13}C -NMR spectrum of the cord factor from TMC 5135 (Table 2) supported the data from the ^1H -NMR, and were in agreement with earlier reports (Nishizawa *et al.*, 2007), including those for different types of mycolates (Schroeder & Barry, 2001; Watanabe *et al.*, 1999).

The overall mycolic acid composition of cord factor from *M. simiae* was estimated from the ^1H -NMR spectra and is shown in Table 3. Notably, the data suggested the existence of high proportions of keto-mycolates in TMC 5135 and ATCC 25275^T, the strains with the most elevated *trans*-/*cis*-cyclopropane ratios (Table 3). α' -Mycolates were abundant in all strains except TMC 5135; on the other hand, the amounts of *trans*-unsaturated α - and keto-mycolates varied among strains and seemed to be particularly prominent in the case of ATCC 25275^T (Table 3). ESI-IT-MS (Fig. 3, Table 4), confirmed the general evidence obtained by ^1H -NMR regarding the mycolic acid composition of cord factor in the strains studied. Thus, α' - (C64–C68), α - (C80–C91) and keto- (C85–C91) mycolates were identified in the mass spectra (Fig. 3). According to previous findings with *M. simiae* (Mederos *et al.*, 2007) and other mycobacteria (Minnikin, 1982; Watanabe *et al.*, 2001), the odd carbon number α -mycolate series (Fig. 1a) mainly contains one *trans* di-substituted cyclopropane ring plus one *cis* di-substituted cyclopropane ring, whereas the even carbon number series (Fig. 1b) has two *cis* di-substituted cyclopropane rings. On the other hand, the odd carbon

Table 3. Whole mycolic acid composition of cord factors from different strains of *M. simiae* as deduced from the ^1H -NMR spectra (see Methods)

Strain	$\alpha/\alpha'/\text{keto}$	<i>trans</i> -Cyclopropane/ <i>cis</i> -cyclopropane	<i>trans</i> double bond/ cyclopropane
TMC 5135	1/0.47/1.16	0.72	0.25
ATCC 25275 ^T	1/0.68/1.37	0.73	0.41
IPK-220	1/0.87/0.59	0.62	0.37
IPK-337	1/0.72/0.67	0.66	0.28
IPK-342	1/0.66/0.85	0.60	0.19

number keto-mycolate series (Fig. 1c) showed one *trans* disubstituted cyclopropane ring. It should be noted, however, that the even carbon number series of α - and keto-mycolates can have a *trans* double bond instead of a cyclopropane (Mederos *et al.*, 2007; Watanabe *et al.*, 2001). Taking into account the intensities of the different (M-H)^{-1/z} ions among the α' -mycolate series, C66:1 was always more abundant, as was C87 in the case of keto-mycolates (Table 4). Nevertheless, differences were detected for α -mycolates, because the odd carbon number series (particularly C87 and C89) predominated in TMC 5135, whereas more equal levels of the odd and even carbon number series were found for ATCC 25275^T, IPK-220, IPK-337 and IPK-342 (Table 4). The general patterns of mycolic acids from delipidated cells were similar to those of cord factors (Table 4). It should be noted that TMC 5135 seems to maintain the same levels of odd and even carbon number α -mycolate series in both cord factor and delipidated cells (57.4 vs 55.3 %) (Table 4), whereas in the remaining strains the content of the odd carbon number α -mycolate series was higher in the cord factor than in delipidated cells (45.6–48.4 vs 33.6–37.2 %) (Table 4).

Induction of α -TNF

Cord factor from strains ATCC 25275^T, IPK-342 and TMC 5135 was able to induce the production of α -TNF in RAW 264.7 cells at all assayed concentrations. The results indicated that α -TNF levels increased slightly with the amount of cord factor in the cases of TMC 5135 and IPK-342. As depicted in Table 5, significant statistical differences were noted among strains at 0.1 μg and at 1.0 μg cord factor, but not at 10 μg . However, overall, the induction of α -TNF could be considered similar for the three compounds examined.

DISCUSSION

Data from the present study reveal the existence of cord factor in all *M. simiae* strains studied. The identification of cord factor was achieved by a combination of several techniques; however, the initial results from ^1H -NMR were rather unexpected, because of the overlap of H-5/H-5' and H-6b/H6'b in TMC 5135 and IPK-220, which was not found in strains ATCC 25275^T, IPK-337 and IPK-342. Our

findings suggest that such overlapping can be explained by a solvent effect (Table 1). Naturally occurring mycolic acids so far examined are 2*R*,3*R* (see for example Quémard *et al.*, 1997); congruent with this fact, the ^1H -NMR spectrum obtained when using deuteropyridine as solvent also favours the hypothesis that mycolic acids of *M. simiae* present the same configuration at these chiral centres. However, as NMR analyses were performed on mixtures of cord factor subtypes, the peculiarity observed could be due to the mycolate composition of the different cord factors. Indeed cord factor of TMC 5135 has been shown to contain a specific combination of mycolic acids in which the odd carbon number series (i.e. *trans*-cyclopropanes) and keto-mycolates prevail. It has been demonstrated that these decorations on the long mero mycolic chain induce conformational modifications on the long mero chain (Villeneuve *et al.*, 2007, 2010). Such conformational changes of mycolic acids could under certain conditions give a different NMR spectrum.

The whole-lipid composition of cord factor in the strains studied was qualitatively similar, and characterized by the presence of α' -, α - and keto-mycolates. Thus, six basic combinations of mycolic acids in cord factor can be predicted, and due to the wide variations in the chain length of the individual mycolates (see Table 4), numerous possible molecular species may exist, as already evidenced for cord factors of *M. tuberculosis* and other species of the genus (Fujita *et al.*, 2005).

As indicated above, a specific combination of α -mycoloyl substituents was found for TMC 5135, in which the odd carbon number series prevailed, unlike other strains, which tended to contain more elevated levels of the even carbon number α -mycoloyl series in their cord factors. Mycolic acids from delipidated cells were, as expected, similar to those found in cord factor, although, again, the same differences with respect to the α -mycolates were detected between TMC 5135 and the other strains examined. Other different characteristics among strains are related to keto- and α' -mycolates, and also to the *trans*-/ *cis*-cyclopropane ratio, although no additional distinctive features of TMC 5135 were found. Variations in the fine structure of mycolic acids within strains of a given species have also been reported in *Mycobacterium* (see for example Minnikin, 1982; Watanabe *et al.*, 2001, 2002), although their biological significance is uncertain.

Table 4. Mycolic acid composition of cord factor and delipidated cells of several strains of *M. simiae*

Relative percentages within each series (referred to 100 %) were calculated directly from the intensities of the different ions $(M-H)^{-1}$ obtained by ESI-TRAP-MS. For a given strain, the MS spectrum consisted of at least 50 scans. Values for TMC 5135 are the mean $\pm$ SD of two independent analyses. $(M-H)^{-1}$ values are given with a maximum error of 800 p.p.m. for α - and keto-mycolates.

Mycolic acid ($M-H$) ⁻¹	Strain									
	TMC 5135		ATCC 25275 ^T		IPK-220		IPK-337		IPK-342	
	Cord factor	Delipidated cells	Cord factor	Delipidated cells	Cord factor	Delipidated cells	Cord factor	Delipidated cells	Cord factor	Delipidated cells
α'										
C64 (941.9)	13.8 $\pm$ 1.7	20.3 $\pm$ 4.7	7.8	11.7	15.8	7.8	13.6	3.9	12.7	18.2
C66 (969.9)	70.6 $\pm$ 1.7	74.4 $\pm$ 0.6	79.6	77.9	73.4	79.7	73.6	90.2	73.6	68.2
C68 (997.9)	15.6 $\pm$ 0.0	5.3 $\pm$ 5.3	12.6	10.4	10.8	12.5	12.8	5.9	13.7	13.6
α										
C80 (1165.0)	2.0 $\pm$ 0.2	1.8 $\pm$ 1.1	1.0	1.6	2.9	2.1	2.2	3.5	1.7	4.2
C82 (1193.0)	6.4 $\pm$ 1.8	8.1 $\pm$ 3.2	5.1	10.1	7.6	10.7	9.8	13.2	10.8	11.5
C84 (1221.0)	13.4 $\pm$ 2.2	16.1 $\pm$ 3.5	13.7	26.1	23.5	23.1	19.4	25.2	18.4	26.8
C86 (1249.0)	11.4 $\pm$ 0.8	11.8 $\pm$ 2.7	18.3	20.5	14.7	19.3	16.0	16.7	12.6	14.7
C88 (1277.0)	7.1 $\pm$ 0.1	5.3 $\pm$ 0.4	11.3	7.4	4.7	6.2	6.2	2.9	5.4	4.7
C90 (1305.0)	2.3 $\pm$ 0.9	1.6 $\pm$ 1.0	4.1	0.7	0.9	1.4	0.8	1.3	2.7	1.0
C83 (1207.0)	2.5 $\pm$ 0.4	3.2 $\pm$ 0.7	1.0	1.6	3.8	2.8	3.1	3.2	2.7	4.2
C85 (1235.0)	10.5 $\pm$ 1.3	11.7 $\pm$ 1.6	5.8	7.8	13.8	9.7	11.5	9.6	5.4	9.9
C87 (1263.0)	21.5 $\pm$ 0.7	25.5 $\pm$ 5.8	16.8	15.6	19.1	15.5	20.4	16.7	17.5	15.7
C89 (1291.0)	18.1 $\pm$ 5.2	13.1 $\pm$ 5.4	16.1	7.4	7.9	7.9	9.5	6.4	16.1	6.3
C91 (1319.1)	4.8 $\pm$ 2.4	1.8 $\pm$ 0.8	6.8	1.2	1.1	1.3	1.1	1.3	6.7	1.0
Keto										
C85 (1251.0)	23.6 $\pm$ 0.8	24.6 $\pm$ 4.6	0.0	27.7	32.3	28.1	26.2	29.6	25.0	33.5
C87 (1279.0)	42.9 $\pm$ 3.1	50.1 $\pm$ 2.3	51.1	46.6	44.5	48.4	42.2	45.3	43.0	51.9
C89 (1307.1)	30.8 $\pm$ 3.8	22.6 $\pm$ 0.7	42.2	24.1	21.4	21.4	27.9	23.2	28.9	13.4
C91 (1335.1)	2.8 $\pm$ 0.1	1.8 $\pm$ 0.5	6.7	1.6	1.7	2.1	3.7	1.9	3.1	1.2
Sum of α odd carbon number series	57.4	55.3	46.5	33.6	45.7	37.2	45.6	37.2	48.4	37.1

Table 5. Induction of α -TNF (pg ml⁻¹) in RAW 264.7 cells by cord factor from several strains of *M. simiae*

The results are expressed as a mean $\pm$ SD of two experiments done in duplicate.

Strain	Amount of cord factor (μ g)			
	0*	0.1†	1‡	10§
TMC 5135	1273.6 $\pm$ 179.3	1901.4 $\pm$ 25.7	1922.5 $\pm$ 55.6	1937.6 $\pm$ 28.3
ATCC 25275 ^T	1273.6 $\pm$ 179.3	2049.0 $\pm$ 21.0	1952.1 $\pm$ 26.3	2000.0 $\pm$ 47.7
IPK-342	1273.6 $\pm$ 179.3	1755.7 $\pm$ 80.3	1827.1 $\pm$ 7.8	1917.3 $\pm$ 29.8

*A single control was done in the same plate for the three strains.

†Significant statistical differences between TMC 5135/ATCC 25275^T ($P < 0.01$), TMC 5135/IPK-342 ($P < 0.001$) and ATCC 25275^T/IPK-342 ($P < 0.001$).

‡Significant statistical differences between TMC 5135/IPK-342 ($P < 0.05$) and ATCC 25275^T/IPK-342 ($P < 0.002$).

§No significant statistical differences were found among strains.

An initial approach to the analysis of the biological activity of cord factor of TMC 5135 was made by studying its capacity for induction of α -TNF, in an attempt to explain part of the immunogenicity reported for this strain (Mederos *et al.*, 2006). The results obtained, however, do not allow us to attribute a special meaning to this molecule, in spite of its particular fine structure, since cord factors from ATCC 25275^T and IPK-342 were able to induce similar levels of α -TNF in RAW 264.7 cells. It has been reported that cord factors from non-pathogenic mycobacteria show granulomatogenic properties (Fujita *et al.*, 2007), and that, in general, this particular activity should operate in an α -TNF-dependent way (Takimoto *et al.*, 2006; Welsh *et al.*, 2008). Differences in toxicity and granulomatogenic activity found for the cord factors of various species of mycobacteria have been related to the chain length of α -mycolates (Fujita *et al.*, 2007). Also, various *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) strains (and cord factors isolated from them), differing in methoxy-mycolate content, vary in their capacity to induce several cytokines, including α -TNF (Hayashi *et al.*, 2009). The relationships of mycolic acids to biological activities are also linked to both cyclopropanation (Glickman *et al.*, 2000; Rao *et al.*, 2005, 2006) and oxygenation (Bhatt *et al.*, 2007; Dao *et al.*, 2008; Dubnau *et al.*, 2000). At present, it is difficult to determine the exact way by which these effects operate, although they probably rely on complex mechanisms of recognition of cord factor in which both trehalose and mycoloyl substituents seem to play a role (Bowdish *et al.*, 2009; Ishikawa *et al.*, 2009; Ozeki *et al.*, 2006; Werninghaus *et al.*, 2009).

Cyclopropanation of mycolic acids has been reported to be critical for the biological activity of cord factor of *M. tuberculosis* and to have a strong influence on several aspects of the virulence of that species (Rao *et al.*, 2005, 2006; Riley, 2006). No data are available for α' -mycolates in this context. These compounds, shorter than α - and oxygenated mycolates and with one *cis* double bond, are roughly equivalent to those found in *Nocardia* and *Rhodococcus* (Minnikin & Goodfellow, 1976), whose cord

factors, being granulomatogenic, may have low toxicity (Ueda *et al.*, 2001) or even produce cachexia (Silva *et al.*, 1988). From the present study, it is unclear how the differences in both cyclopropanation and *cis* unsaturation noted among the strains can affect the different levels of induction of α -TNF, in spite of the significant statistical differences noted. Overall, the relationship of this finding to the structural details of the respective cord factors is unclear and may merely represent an experimental artefact.

Irrespective of differences in methodology among different reports, the levels of induction of α -TNF by cord factor from *M. simiae* appear lower than those published for cord factor from wild strains of *M. tuberculosis* (Dao *et al.*, 2008; Rao *et al.*, 2005, 2006), although significantly higher than those from BCG strains and non-pathogenic mycobacteria (*Mycobacterium phlei*) (Hayashi *et al.*, 2009). α -Mycolic acids from *M. tuberculosis* are essentially *cis*-cyclopropanated (Minnikin, 1982; Watanabe *et al.*, 2001). However, the low amounts of *trans* derivatives present in this species seem to play an important role in the virulence properties of this micro-organism, because the deletion of the *cmaA2* gene, responsible for the synthesis of the proximal *trans*-cyclopropane in keto- and methoxy-mycolates (see Takayama *et al.*, 2005), produces a hypervirulence phenotype (Rao *et al.*, 2006), characterized by a high induction of α -TNF. Nevertheless, the *mmaA4* mutant, devoid of oxygenated mycolic acids (see Takayama *et al.*, 2005), also induces high levels of α -TNF and interleukin (IL)-12, and shows reduced virulence (Dao *et al.*, 2008). Other factors, such as proximal *cis*-cyclopropanation in α -mycolic acids, also influence the capacity of the *M. tuberculosis* cord factor to induce the above-mentioned cytokine (Rao *et al.*, 2005).

Strain 'habana' TMC 5135 is considered immunogenic in experimental tuberculosis (Gupta *et al.*, 1979) and leprosy (Singh *et al.*, 1989, 1997), producing in both cases delayed-type hypersensitivity and lymphostimulatory responses. Several attempts have been made to define the antigens responsible for immunoprotection. In this context it has

been found that secretory proteins from 30 to 60 kDa induce IL-2, IL-12 and interferon gamma in macrophages from vaccinated mice (Divya Jyothi *et al.*, 2000). These molecules, together with others such as α -TNF, are important effectors in the immunology of tuberculosis (Cooper, 2009; Dietrich & Doherty, 2009). Because the induction of this cytokine can be achieved by the cord factor of TMC 5135, it could be argued that this molecule should play some role in the immunoprotection against experimental tuberculosis and leprosy observed by several authors (reviewed by Mederos *et al.*, 2006). It remains open to further analysis whether cord factor from TMC 5135 also has a role in the induction of other immunomodulatory proteins (e.g. IL-2 and IL-12).

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