Cyclopropanation of α-mycolic acids is not required for cording in *Mycobacterium brumae* and *Mycobacterium fallax*

Cecilia Brambilla,1 Alejandro Sánchez-Chardi,2 Miriam Pérez-Trujillo,3 Esther Julián1 and Marina Luquin1

1Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
2Servei de Microscòpia Electrònica, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
3Servei de Ressonància Magnètica Nuclear, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain

The capacity to form microscopic cords (cording) of *Mycobacterium* species has been related to their virulence. The compounds responsible for cording are unknown, but a recent study has shown that cording could be related to the fine structure of α-mycolic acids. This investigation attributes the need for a proximal cyclopropane in α-mycolic acids for cording in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG and proposes cyclopropanases as good targets for new chemotherapeutic agents. As other *Mycobacterium* species in addition to *M. tuberculosis* and *M. bovis* form microscopic cords, it would be of major interest to know whether the relationship between proximal cyclopropanation of α-mycolic acids and cording could be extended to non-tuberculous mycobacteria. In this study, we have examined the correlation between the cording and cyclopropanation of α-mycolic acids in two species, *Mycobacterium brumae* and *Mycobacterium fallax*. Scanning electron microscopy images showed, for the first time to our knowledge, the fine structure of microscopic cords of *M. brumae* and *M. fallax*, confirming that these two species form true cords. Furthermore, NMR analysis performed on the same cording cultures corroborates the absence of cyclopropane rings in their α-mycolic acids. Therefore, we can conclude that the correlation between cording and cyclopropanation of α-mycolic acids cannot be extended to all mycobacteria. As *M. brumae* and *M. fallax* grow rapidly and have a simple pattern of mycolic acids (only α-unsaturated mycolic acids), we propose these two species as suitable models for the study of the role of mycolic acids in cording.

INTRODUCTION

*Mycobacterium tuberculosis* and *Mycobacterium bovis* are the principal agents responsible for tuberculosis in humans and domestic and wild animals (O’Reilly & Daborn, 1995; WHO, 2010). Both form microscopic cords, a phenotypic characteristic that has been related to their virulence (see Glickman, 2008). Microscopic cords are snake-like structures formed by the end-to-end and side-to-side aggregation of bacilli in which the orientation of the long axis of each cell is parallel to the long axis of the cord (Koch, 1982) (see Fig. 2).

Studies relating cording with virulence began in 1947 with Middlebrook *et al.* (1947). These authors compared the virulent H37Rv and avirulent H37Ra *M. tuberculosis* strains and found that the formation of cords only occurred in the virulent strain. Later, other researchers working with natural or constructed mutants of *M. tuberculosis*, *M. bovis*, *M. marinum* and *M. abscessus* reported a decrease in the virulence of mutants that lost cording (Bhatt *et al.*, 2007; Dong *et al.*, 2012; Gao *et al.*, 2003, 2004; Glickman *et al.*, 2000; Howard *et al.*, 2006; Makinoshima & Glickman, 2005). In addition, we have demonstrated an increase in the virulence of natural mutants of *M. vaccae*, *M. chubuense*, *M. gilvum*, *M. obuense* and *M. parafortuitum* that had acquired the capacity to form microscopic cords (Julián *et al.*, 2010). So, there is no doubt that this microscopic structure is a virulence factor.

As virulence factors can be targets for selective chemotherapeutic drugs, it is of interest to know what compounds are involved in the formation of cords. In this regard, Bloch *et al.* (1953) isolated a toxic glycolipid from *M. tuberculosis* and related this to the virulence of the tubercle bacillus and
the capacity to form microscopic cords. Bloch named this glycolipid cord-factor, which was later identified as 6, 6′-di-O-mycoloyl trehalose (or trehalose dimycolate, TDM) (Noll et al., 1956). However, subsequent studies demonstrated that non-cording mycobacterial strains also contained TDM, so the mere presence of TDM is insufficient for cord formation (Glickman, 2008).

Recent studies performed with genetically defined mutants have shown that cording could be related to the fine structure of mycolic acids (Glickman et al., 2000; Glickman, 2008). These lipids are 3-hydroxy, 2-alkyl branched high-molecular-mass fatty acids found in the cell wall of all mycobacteria, covalently bound to the arabinogalactan and esterifying trehalose molecules to form TDM or trehalose monomycolate (Marrakchi et al., 2008). The presence of glycerol molecules esterified with mycolic acids in the cell wall of M. smegmatis (Chen et al., 2006) has also recently been reported, as well as the release of free mycolic acids from TDM to form a biofilm matrix in M. tuberculosis and M. smegmatis strains (Ojha et al., 2008, 2010). Seven types of mycolic acid have been described to date. Five of these contain oxygenated functions (methoxy, ω-1 methoxy, keto, epoxy and carboxy) in addition to the hydroxyl and carboxyl groups present in all types of mycolic acid. The other two, named ω- and ω′-mycolic acids, are devoid of any additional oxygenated function and differ in their chain length, containing between 70 and 90 and between 50 and 70 carbon atoms, respectively (Marrakchi et al., 2008).

ω-Mycolic acids are present in all mycobacterial species and possess two cyclopropane rings, one cyclopropane ring plus one double bond, or two double bonds (Marrakchi et al., 2008). M. tuberculosis and M. bovis contain ω-, methoxy- and keto-mycolic acids. Glickman et al. (2000) identified a mycobacterial gene, pcaA, that was required for cording and synthesis of the proximal cyclopropane ring in ω-mycolic acids of both M. tuberculosis and M. bovis BCG (Bacille Calmette–Guérin) (Fig. 1c). M. bovis BCG is an attenuated strain of M. bovis. Its attenuation is the result of multiple genetic deletions; however, M. bovis BCG retains the ability to replicate within humans and mice, and exhibits cording (Behr et al., 1999; Glickman et al., 2000; Hsu et al., 2003; Makinoshima & Glickman, 2005; Pym et al., 2002). Glickman et al. (2000) showed that M. bovis BCG and M. tuberculosis pcaA non-cording mutants contained a double bond in the proximal position of their ω-mycolic acids rather than the cyclopropane ring present in wild cording strains. The altered cording morphologies of the pcaA mutants were restored when pcaA was reintroduced (Glickman et al., 2000). So, in this excellent study, a direct relationship was shown between cording and proximal cyclopropanation of ω-mycolic acids. Accordingly, M. marinum, a species phylogenetically related to M. tuberculosis, forms microscopic cords and exhibits a cyclopropane ring in the proximal position of ω-mycolic acids, like M. tuberculosis and M. bovis BCG (Fig. 1d) (Daffé et al., 1991; Staropoli & Branda, 2008).

Nevertheless, we previously described the presence of cords in M. brumae, a species that does not contain cyclopropanes in its ω-mycolic acids (Luquin et al., 1993). Furthermore, microscopic cords were previously described in M. fallax, another species devoid of

\[ \begin{align*}
(a) & \quad \text{CH}_3-(\text{CH}_2)_a-\text{CH}-\text{CH}-(\text{CH}_2)_b-\text{CH}-(\text{CH}_2)_c-\text{CH}-(\text{CH}_2)_d-\text{CH}_3 \\
& \quad \text{a}=15, \text{b}=12, \text{c}=17, \text{d}=19
\end{align*} \]

\[ \begin{align*}
(b) & \quad \text{CH}_3-(\text{CH}_2)_a-\text{CH}-\text{CH}-(\text{CH}_2)_b-\text{CH}-(\text{CH}_2)_c-\text{CH}-(\text{CH}_2)_d-\text{CH}_3 \\
& \quad \text{a}=15,17, \text{b}=15,17, \text{c}=15,17,19, \text{d}=19,21
\end{align*} \]

\[ \begin{align*}
(c) & \quad \text{CH}_3-(\text{CH}_2)_a-\text{CH}-\text{CH}-(\text{CH}_2)_b-\text{CH}-(\text{CH}_2)_c-\text{CH}-(\text{CH}_2)_d-\text{CH}_3 \\
& \quad \text{a}=19, \text{b}=14, \text{c}=11, \text{d}=23
\end{align*} \]

\[ \begin{align*}
(d) & \quad \text{CH}_3-(\text{CH}_2)_a-\text{CH}-\text{CH}-(\text{CH}_2)_b-\text{CH}-(\text{CH}_2)_c-\text{CH}-(\text{CH}_2)_d-\text{CH}_3 \\
& \quad \text{a}=17,19, \text{b}=10,12, \text{c}=15,17, \text{d}=21
\end{align*} \]
cyclopropane rings (Lévy-Frébault et al., 1983; Rafidinarivo et al., 1985) (Fig. 1a and b). The *Mycobacterium* genus contains more than 100 environmental species that can cause opportunistic infections in humans, and the presence of cords has been described in some of them (Clement et al., 2011; Howard et al., 2006; Julián et al., 2010; Lévy-Frébault et al., 1983; Luquin et al., 1993; Sánchez-Chardi et al., 2011; Staropoli & Branda, 2008). It is of major interest to know whether the relationship between proximal cyclopropanation of α-mycolic acids and cording is common in all mycobacteria or is restricted to *M. tuberculosis* and *M. bovis* BCG since cyclopropane synthases represent attractive drug targets, as humans do not synthesize cyclopropanated fatty acids (Glickman et al., 2000). We therefore decided to reinvestigate whether *M. brumae* and *M. fallax* formed true cords.

One of the problems with cording studies is the correct interpretation of cording morphology. The routine procedure is the observation of cords using light microscopy. However, using this technique it is sometimes difficult to distinguish between cording and clumping, which is a general property of mycobacteria due to their hydrophobic surface. We have developed an easy and reproducible method using scanning electron microscopy (SEM) that enables the observation of the ultrastructure of mycobacterial aggregates and differentiation between cords and clumps (Julián et al., 2010).

**Fig. 2.** Representative electrographs of SEM at varying magnifications (a) and photographs of Ziehl-Neelsen stains (b). (i) *M. brumae*, (ii) *M. fallax*, (iii) *M. bovis* BCG and (iv) *M. marinum*. 
So, in this study, we have used SEM to reinvestigate the formation of cords in *M. brumae* and *M. fallax*. Furthermore, the absence of cyclopropane rings in the α-mycolic acids of these species has been corroborated by NMR spectroscopy analysis.

METHODS

**Bacterial strains and growth conditions.** *M. brumae* ATCC 51384T, *M. fallax* ATCC 35219T, *M. bovis* BCG ATCC 35737T Japan strain and *M. marinum* ATCC 927T were grown in Middlebrook 7H9 broth (Difco), supplemented with 10% albumin-glucose-catalase enrichment (Difco) and 0.5% (v/v) glycerol. Cultures were incubated for 4 weeks at 30 °C, except for *M. bovis* BCG which was incubated at 37 °C for 6 weeks.

**Light microscopy and scanning electron microscopy (SEM).** Ziehl-Neelsen stains of spreading pellicles formed by mycobacteria on 7H9 were observed with a Leica-DMRBE microscope (Leica) equipped with differential interference contrast. Micrographs were taken using a DC 250 digital camera system. Cells for SEM evaluation were taken from spreading pellicles, fixed in 2.5% (v/v) glutaraldehyde (EM grade, Merck) in phosphate buffer (PB; 0.1 M, pH 7.4; Sigma-Aldrich) overnight at 4 °C. After four 10 min washes in PB, samples were post-fixed in 1% (w/v) osmium tetraoxide (TAAB Lab) in PB for 2 h at 4 °C, washed four times for 10 min in water, dehydrated in a graded ethanol series (30, 50, 70, 90, 95 and 100%) and dried by critical point drying with CO2. Samples were then mounted on metallic stubs with adhesive carbon films, coated with gold and observed with an S-570 scanning electron microscope (Hitachi Ltd.) operating at 15 kV.

**Extraction and purification of α-mycolic acids.** For extraction and methylation of mycolic acids, cells from spreading pellicles were subjected to an acid methanolysis procedure (Minnikin et al., 1980). Briefly, cells were treated with methanol, toluene and sulphuric acid (30:15:1 by vol.). The mixture was heated at 80 °C for 16 h and the samples were extracted twice with 6 ml n-hexane. The n-hexane samples containing the methyl mycolates were evaporated to dryness at 40 °C under a stream of nitrogen. The mycolates were concentrated by precipitation in cold methanol and analysed by conventional TLC on silica gel-coated plates (G-60, Merck). Plates were developed with n-hexane-diethyl ether (85:15 by vol.). The separate spots were revealed as dark spots by spraying with 10% (w/v) molybdophosphoric acid (Merck) in ethanol followed by charring at 120 °C. Purification of α-mycolates was performed by preparative TLC using plates with a concentration zone (G-60, Merck) developed as described above. Mycolates were visualized with iodine vapours, scraped from TLC plates and recovered with diethyl ether.

**NMR spectroscopy analysis.** Ether extracts containing purified α-mycolates were analysed by NMR spectroscopy. The dried samples were dissolved in 0.6 ml CDCl3 (99.8% D, Cortecnet) and transferred to 5 mm diameter NMR tubes. NMR experiments were performed on a Bruker Avance II 600 (NMR) spectrometer (Bruker Biospin) equipped with a 5 mm TBI probe with Z-gradients and operating at an 1H-NMR frequency of 600.13 MHz. The temperature was set to 298.0 K for all experiments. 1D 1H-NMR spectra were acquired using a standard 90° pulse sequence, with an acquisition time of 1.71 s and a relaxation delay of 2 s. The data were collected into 32768 computer data points, with a spectral width of 9590 Hz and as the sum of 128 transients. The resulting free induction decays were Fourier transformed, manually phased and baseline corrected. The homonuclear 2D NMR experiment 1H-1H COSY (correlation spectroscopy) was performed using a standard pulse sequence (Bruker library) and acquired under routine conditions in order to confirm the assignments. All the spectra were calibrated using the residual solvent signal (CHCl3, 7.27 p.p.m.). Chemical shift data are expressed in p.p.m. and coupling constant (J) values in Hz. Multiplicity of peaks is abbreviated as d (doublet), t (triplet) and ddd (double doublet of doublets).

**RESULTS**

All the studied strains grew on 7H9 liquid medium, forming spreading pellicles. Ziehl-Neelsen smears of the spreading pellicles showed cord formation in *M. brumae*, *M. fallax*, *M. bovis* BCG and *M. marinum*, in accordance with previous descriptions (Fig. 2b) (Lévy-Frébault et al., 1983; Luquin et al., 1991; Uenishi et al., 1993; Staropoli & Branda, 2008; Sánchez-Chardi et al., 2011). SEM images of these spreading pellicles revealed the formation of microscopic cords in all cases (Fig. 2a). Using high magnification, we can clearly observe the typical ultrastructure described for true cords, i.e. the arrangement of the bacilli end-to-end and side-to-side in parallel along the long axis of the cord. Thus, *M. brumae* (Fig. 2, i) and *M. fallax* (Fig. 2, ii) aggregates are true cords and not clumps. The ultrastructures of *M. brumae* and *M. fallax* cords were very similar to the ultrastructures of cords formed by the control strains *M. bovis* BCG and *M. marinum* (Fig. 2, iii and iv, respectively).

Mycolate TLC analysis showed the previously described pattern, i.e. only α-mycolates for *M. brumae* and *M. fallax* (Fig. 3, lanes 2 and 3, respectively) (Luquin et al., 1993; Rafidinarivo et al., 1985), and α-, methoxy- and ketomycolates for *M. bovis* BCG and *M. marinum* (Fig. 3, lanes 1 and 4, respectively) (Daffe et al., 1991; Uenishi et al., 2008).

![Fig. 3. 1D TLC of mycolic acid methyl esters. Lanes: 1, *M. bovis* BCG; 2, *M. brumae*; 3, *M. fallax*; 4, *M. marinum*. TLC was developed three times with n-hexane/diethyl ether (85:15 by vol.) and revealed with molybdophosphoric acid.](image-url)
1H-NMR spectra of \textit{M. brumae} and \textit{M. fallax} \(\alpha\)-mycolates (Fig. 4a and b) showed signals attributed to olefinic protons corresponding to \textit{cis} double bonds, multiplets at a chemical shift of 5.35 p.p.m., and \textit{trans} double bonds, multiplets at chemical shifts of 5.34 and 5.24 p.p.m. \(\text{\textsuperscript{1}}\text{H}\)-NMR signals indicating cyclopropane (cp) rings: 0.65, 0.56 and 0.34 p.p.m. The numbered peaks correspond to the numbered hydrogens. All \(\text{\textsuperscript{1}}\text{H}\)-NMR spectra showed the characteristic peaks of \(\alpha\)-mycolic acid, such as a methyl ester singlet at 3.72 p.p.m., a \(\beta\)-carboxylic proton at 3.66 p.p.m., an \(\alpha\)-carboxylic proton at 2.44 p.p.m., methylene protons adjacent to the \(\alpha\)-carboxylic group at 1.71 p.p.m., methylene chains at 1.27 p.p.m. (broad), branched methyl group at 0.95 p.p.m. and a terminal methyl group resonating at 0.89 p.p.m. See text for more information.

\(\text{\textsuperscript{1}}\text{H}\)-NMR spectra of \textit{M. brumae} and \textit{M. fallax} \(\alpha\)-mycolates (Fig. 4a and b) showed signals attributed to olefinic protons corresponding to \textit{cis} double bonds, multiplets at a chemical shift of 5.35 p.p.m., and \textit{trans} double bonds, multiplets at chemical shifts of 5.34 and 5.24 p.p.m. The peak corresponding to protons adjacent to a double bond was also identified at 1.96 p.p.m. (Yuan & Barry, 1996). No \(\text{\textsuperscript{1}}\text{H}\)-NMR signals corresponding to cyclopropane rings (\textit{cis}: 0.65, 0.56 and 0.34 p.p.m. or \textit{trans}: 0.45, 0.19, 0.13 and 0.09 p.p.m.) (Watanabe \textit{et al.}, 1999) were observed in \textit{M. brumae} or in \textit{M. fallax} (see Fig. 4a and b) in accordance with the previous description by Luquin \textit{et al.} (1993) and Rafidinarivo \textit{et al.} (1985). In the case of \textit{M. bovis} BCG, typical signals corresponding to \textit{cis} cyclopropane rings were observed at 0.65 (multiplet, broad), 0.56 (ddd, 8.2, 8.2, 4.2 Hz) and 0.34 (ddd, 4.2, 5.3, 5.3 Hz) p.p.m. \(\text{\textsuperscript{1}}\text{H}\)-NMR peaks assigned to protons adjacent to the cyclopropane ring, multiplets at 1.38 and 1.15 p.p.m. were also identified (Fig. 4c) (Watanabe \textit{et al.}, 1999). The \(\text{\textsuperscript{1}}\text{H}\)-NMR spectrum of \textit{M. bovis} BCG did not show any signal for double bonds (5.35, 5.34 and 5.24 p.p.m.), in accordance with the reported structures of \(\alpha\)-mycolic acids for this strain (Fig. 4c) (Uenishi \textit{et al.}, 2008). Signals corresponding to \textit{cis} cyclopropane rings and \textit{trans} double bonds could be seen in \(\alpha\)-mycolates of \textit{M. marinum} (Fig. 4d), as previously reported by Daffe \textit{et al.} (1991). All \(\text{\textsuperscript{1}}\text{H}\)-NMR spectra showed the characteristic peaks of \(\alpha\)-mycolic acid, such as a methyl ester singlet at 3.72 p.p.m., a \(\beta\)-carboxylic proton at 3.66 p.p.m., an \(\alpha\)-carboxylic proton at 2.44 p.p.m., methylene protons adjacent to the \(\alpha\)-carboxylic group at 1.71 p.p.m., methylene chains at 1.27 p.p.m. (broad), branched methyl group at 0.95 p.p.m. and a terminal methyl group resonating at 0.89 p.p.m. (Yuan & Barry, 1996). These signals are shown in Fig. 4(a) as an example.`
DISCUSSION

The compounds responsible for the formation of cords in mycobacteria are currently unknown, but studies performed with genetically defined mutants have provided evidence that mycolic acids could be involved in cording. Alterations in biosynthesis, chain length and functional groups of mycolic acids in mutants of M. bovis BCG, M. tuberculosis and M. marinum caused the loss of the cording phenotype (Dong et al., 2012; Glickman et al., 2000). The need for the presence of a cyclopropane ring in the proximal position of α-mycolic acids for cording was established in one of these studies (Glickman et al., 2000).

In the original description of M. brumae and M. fallax, it was reported that these two species formed microscopic cords; however, both were devoid of cyclopropane rings in their α-mycolic acids (Lévy-Frébault et al., 1983; Luquin et al., 1993; Rafidinarivo et al., 1985). As these data disagree with the result obtained with M. tuberculosis and M. bovis BCG mutants (Glickman et al., 2000), we decided to reinvestigate whether M. brumae and M. fallax formed true cords.

The results obtained from this study demonstrate that M. brumae and M. fallax form true cords and confirm that both species contain α-mycolic acids devoid of cyclopropane rings. We have therefore clearly demonstrated that the presence of a cyclopropane ring in the proximal position of α-mycolic acids is not necessary for cording in M. brumae and M. fallax. A direct implication of this conclusion is that drugs targeting cyclopropane synthases could be useful for the treatment of tuberculosis, but not for the treatment of other illnesses produced by non-tuberculous mycobacteria.

M. brumae and M. fallax only have α-mycolic acids; however, M. tuberculosis contains α-, methoxy- and ketomycolic acids, and the M. bovis BCG strain used by Glickman et al. (2000) contains α- and keto-mycolic acids. So, there is no single structure and pattern of mycolic acids responsible for cording in mycobacteria. This is one of the major conclusions of our study.

However, more studies are needed in order to demonstrate that mycolic acids are directly involved in the formation of microscopic cords. In this regard, M. fallax and M. brumae are excellent models for the study of the correlation between mycolic acids and the formation of microscopic cords, as they grow rapidly and contain a simple pattern of mycolic acids.

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