Temperature-induced changes in the cell-wall components of *Mycobacterium thermoresistibile*

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The mycobacterial cell wall consists of a core composed of peptidoglycan linked to the heteropolysaccharide arabinogalactan, which in turn is attached to mycolic acids. A variety of free lipids complements the mycolyl residues, whereas phosphatidylinositol mannosides (PIMs), lipoarabinomannan and proteins are interspersed in this framework. As a consequence, the cell envelope is extremely rich in lipids and early work has shown that the lipid content may vary with environmental conditions. To extend these studies, the influence of growth temperature on cell envelope components in *Mycobacterium thermoresistibile*, a temperature-resistant mycobacterial species, was investigated. Mycolic acid synthesis was reduced at 55 °C compared to 37 °C and the production of fatty acids, presumably precursors of mycolic acids, was increased. Since fatty acids are elongated by the type II fatty acid synthase complex and consequently by a mycobacterial β -ketoacyl acyl carrier protein synthase (KasA), leading to mycolic acids, the expression level of KasA was analysed by Western blotting. KasA expression was significantly decreased at 55 °C over 37 °C. Important changes in the mycolic acid composition were observed and characterized by reduced levels of cyclopropanation and the concomitant accumulation of the *cis*-olefin derivatives. In addition, striking differences involved in complex lipid composition, including acylated trehaloses and trehalose dimycolate (TDM) were also observed. At 55 °C, *M. thermoresistibile* produced less TDM than at 37 °C, which could be explained by the down-regulation of antigen 85 (Ag85) expression as shown by Western blotting. The Ag85 complex represents a family of proteins known to catalyse the transfer of mycolates to trehalose, thereby generating TDM. Furthermore, at 55 °C the level of phosphatidylinositol hexamannoside (PIM₆) synthesis, but not that of other PIM species, was dramatically reduced. This observation could be correlated to a decrease of mannosyltransferase activity associated with membranes prepared from cells grown at 55 °C as compared to 37 °C. Altogether, this study suggests that mycobacteria are capable of inducing important cell-wall changes in response to temperature variations, which may represent a strategy developed by the bacteria to adapt to environmental changes.

Keywords: mycobacteria, fatty acid synthase II, mycolic acid, KasA, temperature

Abbreviations: AcpM, mycobacterial acyl carrier protein; AG, arabinogalactan; FAME, fatty acid methyl ester; FAS, fatty acid synthase; KasA, β -ketoacyl AcpM synthase A; LAM, lipoarabinomannan; LM, lipomannan; MAME, mycolic acid methyl ester; PIM, phosphatidylinositol mannoside; PPM, polyphosphoryl mannoside; TDM, trehalose dimycolate.

INTRODUCTION

The mycobacterial cell envelope and its associated components have been implicated as direct modulators of key interactions between mycobacteria and their environment (Daffé & Draper, 1998). However, very little is known about the mechanisms by which mycobacteria modulate expression of cell-wall components in response to changes in their environment, such as oxidative stress, growth, anaerobiosis, nutrient deprivation and temperature shifts. In bacteria, optimization of membrane function at various temperatures depends on a close regulation of membrane fluidity (Magnuson *et al.*, 1993). For instance, *Escherichia coli* adjusts its fatty acid composition in response to lower temperature by increasing the amount of *cis*-vacenic acid and decreasing the amount of palmitic acid incorporated into membrane phospholipids (Marr & Ingrahm, 1962).

The mycobacterial cell envelope differs substantially from the canonical cell-wall structures of Gram-negative and Gram-positive bacteria (Brennan & Nikaido, 1995). In addition to the typical cell-membrane bilayer and peptidoglycan layers found in other bacteria, the mycobacterial cell envelope contains mycolic acids covalently attached to arabinogalactan (AG) and a wide range of non-covalently attached complex lipids and glycolipids (Minnikin, 1982). Some of these lipids also exhibit potent biological activities and thus may be considered as important virulence factors (Cox *et al.*, 1999; Camacho *et al.*, 2001). Interestingly, some of them are exported and can be found within the cytoplasm of host macrophages, dissociated from the intact bacilli (Beatty *et al.*, 2000). It has been proposed that the chemical diversity of lipids and glycolipids found in the mycobacterial cell envelope mediates specific interactions with host ligands or membranes rather than being solely responsible for the hydrophobicity and rigidity of the mycobacterial cell wall.

The capacity of pathogenic mycobacteria to survive and replicate within the hostile environment of the host macrophage depends on the existence of adaptive mechanisms of bacterial cell physiology. Although environmental adaptation of the mycobacterial cell wall and its components remains largely unknown, it is very likely that they participate in the adaptive process. The first evidence for this kind of regulatory process came from work conducted by Davidson *et al.* (1982) in *Mycobacterium microti*, a close relative of *Mycobacterium tuberculosis*. It was shown that young growing cultures harvested from mouse lungs contained high proportions of ketomycolates, whereas stationary phase cultures had roughly equal proportions of keto- and methoxymycolates. The proportion of α -mycolates increased slightly with the age of the culture, but was always less than one-third of the total mycolate content. More recently, Yuan *et al.* (1998) reported that ketomycolates were produced more predominantly during intracellular growth of *M. tuberculosis* within THP-1 macrophages. These authors also demonstrated that the ratio of methoxymycolates to ketomycolates was altered

by oxygen tension and that the ratio produced under low oxygen tension was identical to that of organisms grown *in vivo*.

In this report, we describe the effect of growth temperature on mycolic acid, fatty acid and complex lipid composition in *Mycobacterium thermoresistibile*. The rationale of the use of this strain is that it survives and replicates at elevated temperatures (up to 55 °C) compared to most mycobacterial species, a property that facilitates the analysis of cell-wall component changes induced by growth at high temperature.

METHODS

Strain and media. *M. thermoresistibile* ATCC 19527 was usually grown at 37 °C under constant shaking in Sauton medium supplemented with 10% OADC (Difco). Cultures grown at 55 °C were obtained by shifting the temperature progressively from 37 °C to 55 °C. Colony-forming units were plated on Middlebrook 7H11 solid media and incubated at 37 °C for 3–4 days.

Mycolic acid and fatty acid profiles of cells grown at high temperature. [1,2-¹⁴C]Acetate [50–62 mCi mmol⁻¹ (1.85–2.29 GBq mmol⁻¹; Amersham] was added at 1 μ Ci ml⁻¹ (37 kBq ml⁻¹) to mycobacterial cultures grown at mid-exponential phase, followed by a further 6 h incubation at either 37 °C or 55 °C. ¹⁴C-Labelled cells were harvested by centrifugation at 2000 *g* and washed once with PBS. Washed cells were then subjected to alkaline hydrolysis using 2 ml 15% tetrabutylammonium hydroxide (TBAH) at 100 °C overnight and mixed with 4 ml CH₂Cl₂, 300 μ l CH₃I and 2 ml H₂O for 1 h. The upper aqueous phase was discarded and the lower organic phase was washed twice with water and dried. The lipids were extracted using diethyl ether, re-dried and resuspended in 200 μ l CH₂Cl₂. An aliquot of the resultant mixture of fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) was then subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄; Merck) developed in petroleum ether/acetone (19:1, v/v). The FAMES were separated by reverse-phase TLC on C₁₈-silica gel plates (Sigma) using chloroform/methanol (2:3, v/v).

In some instances, the individual MAMES were resolved through two-dimensional silver ion argentation TLC by immersing 80% of a square silica gel TLC plate into a 10% (w/v) aqueous silver nitrate solution. Following air-drying, plates were activated at 100 °C for 1 h. ¹⁴C-Labelled samples were run in the first dimension along the narrow strip without silver impregnation by developing twice with hexane/ethyl acetate (19:1, v/v). The plates were then dried, turned 90 degrees and run into the silver layer by developing three times with petroleum ether/diethyl ether (17:3, v/v). Autoradiograms were obtained by exposure to Kodak X-Omat AR film to reveal [¹⁴C]FAMES and [¹⁴C]MAMES.

Systematic analysis of complex mycobacterial lipids. The lipid composition was analysed using [1,2-¹⁴C]acetate-labelled *M. thermoresistibile* cells. Lipids were extracted by adding 2 ml CH₃OH/0.3% NaCl (10:1, v/v) and 1 ml petroleum ether to pelleted cells. After centrifugation, the upper petroleum ether layer was removed and 1 ml petroleum ether was added. The combined petroleum ether extracts were then evaporated under nitrogen to yield apolar lipids that were resuspended in CH₂Cl₂ prior to TLC analysis. For extraction of polar lipids, the methanolic saline extract was heated at

65 °C for 5 min and 2.3 ml CHCl₃/CH₃OH/0.3 % NaCl (9:10:3, by vol.) was added to the extract and mixed on a tube rotator for 10 min. The solvent extract was then separated from the biomass by centrifugation and the supernatant retained. The pellet was further extracted with 0.75 ml CHCl₃/CH₃OH/0.3 % NaCl (5:10:4, by vol.) for 10 min. The combined solvent extracts were mixed with 1.3 ml CHCl₃ and 1.3 ml 0.3 % NaCl for 5 min. After centrifugation the lower organic layer was collected and evaporated to dryness to yield the polar lipids which were resuspended in CHCl₃/CH₃OH/H₂O (10:10:3, by vol.) prior to TLC analysis.

The complex lipids were analysed by two-dimensional TLC using a variety of solvent systems. Five systems were necessary to cover the polarity range of both non-polar and polar mycobacterial lipids (Besra, 1998): briefly, system A, first dimension, petroleum ether/ethyl acetate 98:2, v/v (×3); second dimension, petroleum ether/acetone (49:1, v/v); system B, first dimension, petroleum ether/acetone 23:2, v/v (×3); second dimension, toluene/acetone (19:1, v/v); system C, first dimension, CHCl₃/CH₃OH (24:1, v/v); second dimension, toluene/acetone (4:1, v/v); system D, first dimension, CHCl₃/CH₃OH/H₂O (100:14:0.8 by vol.); second dimension, CHCl₃/acetone/CH₃OH/H₂O (50:60:2.5:3 by vol.); and system E, first dimension, CHCl₃/CH₃OH/H₂O (10:5:1, by vol.); second dimension, CHCl₃/acetic acid/CH₃OH/H₂O (40:25:3:6, by vol.). Lipids were detected by exposure of the TLCs to Kodak X-Omat AR film and charred at 110 °C following spraying with α -naphthol or 5 % molybdo-phosphoric acid.

Anti-KasA immune serum. Recombinant His-tagged β -ketoacyl mycobacterial ACP (AcpM) synthase (KasA) protein was produced in *E. coli* carrying pET28a-kasA and purified by affinity chromatography using a His-Trap column (Pharmacia) as described previously (Kremer *et al.*, 2002a). Purified KasA was used to prepare an anti-KasA immune serum. A rat was injected four times with 25 μ g KasA mixed with an equal volume of adjuvant (monophosphoryl lipid A + trehalose dicorynomycolate; Sigma) at 1, 15, 30 and 60 days. The serum was collected at different time points after the last injection and the specificity of the antibodies was tested by Western blotting.

Electrophoresis and Western blot analyses. *M. thermoresistibile* cells were harvested, resuspended in PBS and disrupted by sonication. Protein concentration was determined on total lysates using the BCA Protein Assay Reagent kit (Pierce) according to the manufacturer's instructions. Twenty micrograms of total protein was subjected to SDS-PAGE as described by Laemmli (1970) with 12 % acrylamide gels on a MiniProtein II system (Bio-Rad). Proteins were either stained with Coomassie blue R350 (Amersham Pharmacia) or transferred to nitrocellulose membranes for Western blot analysis (Kremer *et al.*, 1995). For detection of KasA, membranes were incubated overnight with the rat anti-serum directed against KasA at a dilution of 1/500, washed and subsequently incubated with anti-rat antibodies conjugated to alkaline phosphatase (Promega) used at a 1/7000 dilution. Detection of the antigen 85 (Ag85) complex was done by probing the membranes with a mixture of monoclonal antibodies 17/4 and 32/4 diluted 1/10 (Huygen *et al.*, 1994). Membranes were then incubated with anti-mouse antibodies coupled to alkaline phosphatase (Promega) and used at a 1/7000 dilution.

Incorporation of radiolabelled mannose (Man) from GDP-[¹⁴C]Man into membrane lipids. The enzymically active membrane fraction of *M. thermoresistibile* was prepared as previously described (Besra *et al.*, 1997). To mannosylate

endogenous polyprenol monophosphate lipid substrates, the membrane fractions were incubated in buffer A [50 mM MOPS (adjusted to pH 7.9 with KOH), 5 mM β -mercapto-ethanol, 10 mM MgCl₂] containing 0.1 mM DTT, 20 mM NaF, 2.4 μ M [0.125 μ Ci (4.62 kBq)] GDP-[U-¹⁴C]mannose [321.4 mCi mmol⁻¹ (11.9 GBq) DuPont NEN] and 62.5 μ M ATP in a total volume of 50 μ l at 37 °C for 30 min. The reaction was terminated by the addition of 4 ml CHCl₃/CH₃OH/0.8 M NaOH (10:10:3, by vol.) and was followed by incubation at 55 °C for 15 min. The reaction mixture was cooled and 1.75 ml CHCl₃ and 0.75 ml H₂O were added. After centrifugation the upper aqueous phase was discarded. The lower phase was washed three times with 2 ml CHCl₃/CH₃OH/H₂O (3:47:48, by vol.) to yield an organic fraction, which contained exclusively the mild-alkali stable family of polyprenol monophosphoryl mannoses (PPMs). This fraction was then dried under a stream of nitrogen and resuspended in 200 μ l CHCl₃/CH₃OH (2:1, v/v). PP[¹⁴C]Ms were quantified by scintillation counting and equal aliquots (4000 c.p.m.) were resolved by TLC on silica gel plates using CHCl₃/CH₃OH/H₂O (65:25:4, by vol.). Autoradiograms were obtained by exposing the chromatograms to Kodak X-Omat AR films for 2–3 days.

To assess the transfer of [¹⁴C]Man from GDP-[¹⁴C]Man to the phosphatidylinositol mannosides (PIMs), membrane fractions were incubated in buffer A containing 0.1 mM DTT, 20 mM NaF, 62.5 μ M ATP, 10 mM CaCl₂ and 2.5 μ g amphomycin (a lipopeptide antibiotic that specifically inhibits polyprenyl-phosphate-requiring translocases and the synthesis of C₃₅/C₅₀-P-Man) in a total volume of 50 μ l. Reactions were incubated for 10 min at 37 °C prior to the addition of 2.4 μ M (0.125 μ Ci) GDP-[U-¹⁴C]Man, and held at 37 °C for a further 50 min. The reaction was stopped by the addition of 4 ml CHCl₃/CH₃OH/H₂O (10:10:3, by vol.) and incubation at room temperature for 30 min, followed by the addition of 1.75 ml CHCl₃ and 0.75 ml H₂O. The lower organic layer of the biphasic mixture was washed three times with 2 ml CHCl₃/CH₃OH/H₂O (3:47:48, by vol.), dried under a stream of nitrogen and resuspended in 200 μ l CHCl₃/CH₃OH (2:1, v/v). The transfer of [¹⁴C]Man from GDP-[¹⁴C]Man to the PIMs was quantified by scintillation counting and the material was analysed by TLC-autoradiography as described previously (Besra *et al.*, 1997).

RESULTS

Growth of *M. thermoresistibile* at high temperature inhibits mycolic acid biosynthesis

Bacteria alter the acyl chains of their membrane phospholipids in response to changes of environmental conditions (Cronan, 2002). The three major acyl-chain modifications all involve double bonds. First, desaturation can occur, where a double bond is introduced into a saturated acyl chain, and second, *cis-trans* isomerization can be observed. Third, in cyclopropane fatty acid synthesis, a methylene carbon may be added across the double bond to form a three-membered ring. We therefore asked whether temperature changes may also alter mycobacterial fatty acid and mycolic acid synthesis. Mycolic acids from *M. thermoresistibile* consist of three main subclasses, designated as α -, methoxy- and ketomycolates, and a minor subclass of α' -mycolates (Levy-Frebault *et al.*, 1986). The α -, methoxy- and keto-subclasses consist of C₇₂–C₈₀ cyclopropanated/dienoic/

Fig. 1. Elevated growth temperature of *M. thermoresistibile* inhibits mycolic acid production and induces accumulation of fatty acids. Mycobacteria were grown in liquid medium either at 37 °C or 55 °C and labelled with 1 μ Ci (37 kBq) [1,2- 14 C]acetate ml $^{-1}$. Labelled lipids were extracted, derivatized and resolved by normal-phase TLC using petroleum ether/acetone (19:1, v/v). Equal counts (200 000 c.p.m.) were loaded in each lane. α , M and K indicate α -, methoxy- and keto-MAMES, respectively.

monoenoic acids, whereas the α' -mycolates are C₆₀-monoenoic acids, due to their shorter meromycolic chain (Kremer *et al.*, 2000). To determine the effect of growth temperature, *M. thermoresistibile* was grown either at 37 °C or at 55 °C, labelled with [14 C]acetate, and both FAMES and MAMES extracted and analysed by TLC and autoradiography. Surprisingly, a temperature shift to 55 °C was associated with a dramatic

decrease in α -, methoxy- and ketomycolate synthesis (Fig. 1). The shorter α' -species cannot be resolved in the solvent system used in Fig. 1 and as a consequence we turned to a more complete resolution of MAME subfamilies using silver ion argentation chromatography as described below.

Temperature alters the mycolic acid profile of *M. thermoresistibile*

To determine whether a temperature shift also alters [14 C]acetate-labelled MAMES, we examined the MAME subfamilies by two-dimensional argentation TLC. This technique has been used previously for the analysis of *M. tuberculosis* MAMES (George *et al.*, 1995; Glickman *et al.*, 2001). Briefly, the sample was developed in the first dimension along the un-impregnated strip to separate the mycolates by polarity. The plate is then developed in the second dimension (silver nitrate) to separate the mycolates by degree and type of unsaturation. Indeed, silver nitrate retards the migration of unsaturated lipids relative to saturated or cyclopropanated lipids. The TLC pattern of *M. thermoresistibile* grown at 37 °C and 55 °C is shown in Fig. 2. At 37 °C, α -, methoxy- (M) and ketomycolates (K) were mainly present as cyclopropanated species along with a lower abundance of α' -mycolates. Minor species with the polarity of α -, methoxy- and ketomycolates but which are retarded by silver impregnation probably correspond to *cis*- or *trans*-olefins. In contrast, at 55 °C, the degree of cyclopropanation was dramatically reduced, with the α -mycolates giving rise to diunsaturated *cis/cis*-mycolates and the methoxymycolates being almost completely replaced by their *cis*-olefin precursor. Interestingly, the level of cyclopropanated ketomycolates remained unaffected. In addition, at 55 °C a complete loss of *trans*-olefins for all mycolic acid subclasses was observed. Thus, elevated growth temperatures appear to result in the accumulation of *cis*-olefins containing

Fig. 2. Two-dimensional silver ion argentation autoradiographic TLC of [1,2- 14 C]acetate-labelled *M. thermoresistibile*. Cultures incubated either at 37 °C (left panel) or 55 °C (right panel) were labelled with [1,2- 14 C]acetate. 14 C-labelled FAMES-MAMES were isolated as described in Methods and 100 000 c.p.m. of each extract applied to two-dimensional silver ion argentation TLC plates. The plates were developed in the first direction by using two developments of hexane/ethyl acetate (19:1, v/v) and in the second direction by using a triple development of petroleum ether/diethyl ether (17:3, v/v). α , α' , M and K indicate α -, α' -, methoxy- and keto-MAMES, respectively. Autoradiograms were obtained after 4 days exposure to Kodak X-Omat AR film.

Fig. 3. Attachment of fatty acids to the cell wall. *M. thermoresistibile* cells were grown in liquid medium at 37 °C or 55 °C and labelled with 1 μ Ci (37 kBq) [1,2-¹⁴C]-acetate ml⁻¹. Following delipidation of the mycobacterial cell wall, FAMES and MAMES were extracted, derivatized and equal numbers of counts subjected to TLC analysis. (A) Normal-phase TLC, 60000 c.p.m. per lane, resolved using petroleum ether/acetone (19:1, v/v) (lower panel). The upper panel represents part of a two-dimensional silver ion argentation autoradiographic TLC illustrating the separation of unsaturated fatty acids (UFA) from saturated fatty acids (SFA). α , M and K indicate α -, methoxy- and keto-MAMES, respectively. (B) Reverse-phase TLC, 40000 c.p.m. per lane, resolved using CHCl₃/CH₃OH (2:3, v/v). Autoradiograms were obtained after exposure to Kodak X-Omat AR film for 3–4 days.

MAMES, concomitant with the lack of cyclopropanated MAMES.

High-temperature growth of *M. thermoresistibile* causes accumulation of fatty acid synthase (FAS-I) end products and decreases of expression of enzymes involved in mycolic acid synthesis and transfer

Since FAS-I is believed to generate FAS-II substrates (Kremer *et al.*, 2000), one would expect that reduction of mycolic acid biosynthesis through FAS-II inhibition would cause accumulation of the FAS-I products. As shown in Fig. 1, growth at 55 °C induces a strong production/accumulation of FAMES that are the end products of FAS-I. The *in vitro* activities of FAS-I from *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG were characterized by Peterson & Bloch (1977) and Kikuchi *et al.* (1992), respectively. A bimodal fatty acid pattern of C₁₆ and C₂₄ was reported for *M. smegmatis*, whilst *M. bovis* BCG possessed a bimodal fatty acid pattern of C₁₆ and C₂₆. Our analysis by reverse-phase TLC of the ¹⁴C-labelled FAMES from *M. thermoresistibile* that accumulated at 55 °C consisted mainly of C₁₆–C₂₄ fatty acids (data not shown).

To investigate whether the FAMES accumulating at 55 °C were associated to the cell wall or were present as free lipids, ¹⁴C-labelled cells were delipidated to extract free lipids. FAMES and MAMES were then isolated and analysed by TLC and autoradiography. As shown in Fig. 3(a) (lower panel), nearly all FAMES from cells incubated at 37 °C were removed after delipidation. In contrast, FAMES were still present in delipidated cells

and associated with isolated cell walls from bacilli grown at 55 °C (Fig. 3a). In addition, two-dimensional argentation TLC clearly showed that both unsaturated and saturated FAMES accumulated at 55 °C and were associated with the cell wall. Reverse-phase TLC showed these FAMES corresponded mainly to C₁₆–C₂₄ fatty acids (Fig. 3b).

We have assumed that the decrease of mycolic acid biosynthesis observed at 55 °C may be linked to down-regulation of FAS-II components. To test this hypothesis, Western blot analysis was performed to analyse KasA expression in *M. thermoresistibile* crude lysates since KasA has been shown to be part of the FAS-II system (Kremer *et al.*, 2002a). Anti-KasA antibodies were generated against recombinant *M. tuberculosis* KasA. As shown in Fig. 4(a), the anti-KasA antibodies recognized the *M. thermoresistibile* protein. However, the KasA expression level was strongly reduced in extracts from cells grown at 55 °C. This result was found to be reproducible in several independent experiments. The genomic organization around the *kasA* locus in *M. tuberculosis* suggests an operon of five genes encoding several FAS-II components, namely *fabD*, *acpM*, *kasA*, *kasB* and *accD6* (Cole *et al.*, 1998). Thus, a temperature shift to 55 °C may also, presumably, down-regulate the expression of the other genes encoded in the *kas* operon.

The Ag85 complex from *M. tuberculosis* consists of three abundantly secreted proteins (FbpA, FbpB, FbpC2) that play a key role in the pathogenesis of tuberculosis (Wiker & Harboe, 1992) and exhibit cell-wall mycolyl-transferase activity. Belisle *et al.* (1997) and Kremer *et al.*

Fig. 4. Expression levels of KasA and the Ag85 complex by Western blot analysis. (A) Crude lysates (20 µg total protein) from *M. thermoresistibile* cultivated at 37 °C or 55 °C were resolved by 12 % SDS-PAGE, transferred to a membrane and probed with a polyclonal rat antiserum raised against the *M. tuberculosis* KasA protein (1/500 dilution). (B) Crude lysates were resolved by 12 % SDS-PAGE, transferred to a membrane and probed for the presence of the Ag85 complex. A mixture of the two monoclonal antibodies 17/4 and 32/4 (1/10 dilution) raised against the *M. tuberculosis* Ag85 complex was used. M, pre-stained molecular standards.

(2002b) demonstrated that all three purified *M. tuberculosis* antigens catalyse the transfer of mycolates to trehalose via a mycolyltransferase exchange process, leading to the formation of 6-trehalose monomycolate (TMM), 6,6'-trehalose dimycolate (TDM) and to the cell wall proper. Since mycolic acid biosynthesis is inhibited at elevated growth temperatures, we would expect a reduced expression of the Ag85 complex. Therefore, expression of the Ag85 complex was investigated by Western blot analysis using monoclonal antibodies directed against the *M. tuberculosis* Ag85 complex (Huygen *et al.*, 1994). As shown in Fig. 4(b), expression was significantly reduced in a crude lysate from cells grown at 55 °C, compared to a lysate from cells grown at 37 °C. These results suggest that elevated temperatures affect the capacity of the cells to transfer mycolates to trehalose, ultimately leading to TDM (see below), presumably through down-regulation of the Ag85 complex.

Temperature effects on other complex lipids

Associated with the mycolic acid layer are a number of polar and apolar lipids which are thought to extend into the outer surface of the cell. This waxy coat of lipids may account for the limited permeability of mycobacteria as well as for their tendency to grow in large clumps and their natural resistance to toxic substances. The profound alteration of fatty acid and mycolic acid production observed at elevated growth temperature led us to investigate the cellular complex lipid profile by *in vivo* labelling and two-dimensional TLC analysis of the bacteria grown either at 37 °C or at 55 °C. As shown in Fig. 5, the production of many cell-wall components, including diacylated trehalose, multiacylated trehaloses and glycerol monomycolate were altered. TDM, also called cord factor (Bloch, 1950), was found to be down-

regulated at elevated temperature. This glycolipid has been widely implicated in characteristic pathogenic features of mycobacterial diseases, including granuloma formation (Bekierkunst, 1968).

Among other complex lipids whose expression was affected at 55 °C were the PIMs (Fig. 5E). The two PIM₆ populations consisting of Ac₄PIM₆ and Ac₃PIM₆ were appreciably synthesized at 37 °C as determined by two-dimensional TLC after charring with α -naphthol. Synthesis of both Ac₄PIM₆ and Ac₃PIM₆ was strongly inhibited at elevated temperature, whereas the expression of the other PIMs was not altered.

Incorporation of GDP-[¹⁴C]Man by *M. thermoresistibile* membranes

Simpler PIMs (PIM₁, PIM₂, PIM₃) originate from phosphatidyl inositol and GDP-Man, but further growth of the linear lipomannan backbone utilizes C₃₅-/C₅₀-P-Man and is amphotycin-sensitive (Besra *et al.*, 1997). Amphotycin treatment specifically disrupts the action of a variety of translocase enzymes, by chelating polyprenyl monophosphates in the presence of Ca²⁺, and inhibits the transfer of a range of monomeric units to polyprenyl-monophosphate carriers (Besra *et al.*, 1997). Amphotycin was therefore used to measure the transfer of radiolabelled Man from GDP-[¹⁴C]Man into membrane lipids. [¹⁴C]Man-labelled products were extracted from enzymically active membrane fractions that were incubated with GDP-[¹⁴C]Man. Incorporation was quantitatively lower with membrane fractions from *M. thermoresistibile* grown at 55 °C compared to bacilli grown at 37 °C, suggesting decreased GDP-Man-dependent mannosyltransferase activities at 55 °C (data not shown). Analysis by TLC and autoradiography of the [¹⁴C]mannolipids formed showed that, with low

protein concentrations (up to $120 \mu\text{g ml}^{-1}$), PIM₂ was poorly synthesized by membranes prepared from cells grown at 55°C (Fig. 6a). In addition, synthesis of PIM₁ was inhibited in membranes from cells grown at 55°C although this product was clearly formed in membranes from cells grown at 37°C . Altogether, these results suggest that at 55°C mannosyltransferases that utilize GDP-Man as the mannose donor for PIM synthesis are inhibited and/or less efficient in catalysing the mannosyltransferase reactions leading to PIMs.

Finally, we analysed the transfer of [^{14}C]Man from GDP-[^{14}C]Man to polyprenyl-monophosphate carriers. The PPM synthase from *M. tuberculosis* is involved in the generation of PPM, which is subsequently utilized as the Man donor in the biosynthesis of LM (lipomannan) and LAM (lipoarabinomannan) (Gurcha *et al.*, 2002). Labelling of membranes from *M. thermoresistibile* grown at 37°C with GDP-[^{14}C]Man preferentially generated C₅₀-P-Man in a dose-response fashion, whereas C₃₅-P-Man was barely produced (Fig. 6b). Interestingly, labelling of membranes from cells grown at 55°C showed a strong inhibition of C₅₀-P-Man formation, compared to membranes from cells grown at 37°C . This result suggests that incubation of the cells at high temperature negatively affects PPM synthase activity responsible for C₅₀-P-Man biosynthesis. Since C₅₀-P-Man is the mannose donor for linear LM and LAM, and utilizes PIM₂ precursors, it could be argued that the observed *in vivo* decrease of the PIM₆ family at 55°C is associated with both a decrease in the synthesis of PIM₂ and the C₅₀-P-Man donor. However, it remains to be established whether this decrease in PPM synthase activity affects the production of LM/LAM within *M. thermoresistibile* cell walls.

DISCUSSION

One can view the mycobacterial cell wall as an array of distinct lipid molecules, each with its own role in modulating the interaction with its host. Early work by Toriyama *et al.* (1978, 1980) reported the effect of growth temperature on mycolic acid composition and synthesis. It was found that an increase in growth temperature of *Mycobacterium phlei* leads to an enhanced mean chain length of mycolic acids. Baba *et al.* (1989) demonstrated that in *M. smegmatis*, the ratio of α -, α' - and epoxymycolates also varies in response to changes in growth temperature. More recently, it was

Fig. 5. Elevated temperature alters complex cellular lipids that are produced in lower quantity at 55°C . *M. thermoresistibile* was grown in liquid medium and labelled with $1 \mu\text{Ci}$ (37 kBq) [$1,2\text{-}^{14}\text{C}$]acetate ml^{-1} . Labelled lipids were extracted as described in Methods. Equal counts (100000 c.p.m. for apolar lipids, and 50000 c.p.m. for polar lipids) for each extract were applied to two-dimensional TLC plates and developed using different solvent systems as described in Methods. DAT, diacylated trehalose; GMM, glycerol monomycolate; MAT, multiacylated trehalose; TDM, trehalose dimycolate; TG, triglyceride; ?, unassigned product. Autoradiograms were obtained after 3–4 days exposure to Kodak X-Omat AR films.

Fig. 6. α -Mannosyltransferase activity in *M. thermoresistibile* membrane extracts. (A) TLC/autoradiography of the *in situ* labelled endogenous PIMs based on the transfer of [14 C]Man from [14 C]GDP-Man to PIMs. Mannosyltransferase assays contained increasing concentrations of *M. thermoresistibile* membrane fractions, [14 C]GDP-Man, as well as amphomycin. After incubation, the reaction was stopped and extraction of [14 C]PIMs was performed as described in Methods. Approximately 20% of the total counts from each assay were applied to TLC and resolved using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65:25:4, by vol.). (B) TLC/autoradiography of the *in situ* labelled endogenous PPMs ($\text{C}_{35}/\text{C}_{50}$ -PPM) based on the transfer of [14 C]Man from [14 C]GDP-Man to $\text{C}_{35}/\text{C}_{50}$ polyphrenol phosphates. Mannosyltransferase assays contained increasing concentrations of membrane proteins. After incubation, the reaction was stopped and [14 C]PPMs were extracted. Approximately 20% of the total counts from each assay reaction were applied to TLC and resolved using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (65:25:0.4:3.6, by vol.). Autoradiograms were obtained after overnight exposure to a Kodak X-Omat film.

demonstrated that a mutant strain of *M. tuberculosis* that no longer synthesizes oxygenated mycolic acids presents profound envelope permeability alterations and appears strongly attenuated in mice (Dubnau *et al.*, 2000). These observations imply that each subclass composition of mycolic acids is regulated and that the subclasses serve discrete functions. Therefore, genes involved in mycolic acid metabolism, and those involved in modification of the mero chain and elongation steps, may be tightly regulated. In this study, we report a temperature-dependent down-regulation of KasA, an enzyme directly involved in the elongation process of the meromycolate chain by condensing malonyl-AcpM with a growing acyl-AcpM chain (Schaeffer *et al.*, 2001; Kremer *et al.*, 2002a). The decrease in KasA expression correlates with a reduction in mycolic acid biosynthetic activity as evidenced by the lower production of mycolates in *M. thermoresistibile* cells grown at 55 °C.

The mycolic acid *S*-adenosylmethionine-dependent methyl transferases are a large family of highly homologous proteins that modify olefinic mycolic acids with cyclopropane rings and methyl branches. The three classes of mycolates, α -, methoxy- and ketomycolates, are all modified with cyclopropane rings and methyl branches within their mero chain. One of these transferases, PcaA, has been shown as essential for *M. tuberculosis* pathogenesis, since a *pcaA* mutant failed to

establish a chronic persistent *M. tuberculosis* infection in mice (Glickman *et al.*, 2000). PcaA is required for the synthesis of the proximal cyclopropane ring of α -mycolates. It was subsequently shown that CmaA2, another cyclopropane synthase, is required for the synthesis of the *trans* cyclopropane rings of both methoxy- and ketomycolates (Glickman *et al.*, 2001). Inactivation of *cmaA2* causes accumulation of unsaturated derivatives of both methoxy- and ketomycolates (Glickman *et al.*, 2001). We show here that elevated growth temperatures significantly affect the distribution of the different mycolic acid subclasses. Basically, growth at 55 °C causes accumulation of unsaturated derivatives of both α - and methoxymycolates as well as loss of *trans*-containing olefins corresponding to oxygenated mycolic acids. It is therefore tempting to speculate that temperature-dependent inhibition of mycolic acid cyclopropane synthases results in the accumulation of unsaturated mycolic acids. In this regard, recent work has demonstrated that the cyclopropane fatty acid synthase from *E. coli* that catalyses cyclopropanation of fatty acids is a short-lived protein *in vivo* and its degradation is dependent on expression of the heat-shock regulon (Chang *et al.*, 2000). However, whether PcaA, CmaA2 or other mycobacterial cyclopropane synthases are regulated by temperature remains to be established. In addition, differences in the geometrical position of the centre (*cis*-olefin, *trans*-olefin or

-cyclopropane) may directly affect cell-wall fluidity. Overexpression of CmaA2 in *M. smegmatis* was shown to be associated with an increase in the melting temperature of the cell wall (George *et al.*, 1995). It has therefore been suggested that cyclopropanation of the proximal double bond decreases fluidity of the cell wall. In addition, cyclopropanated fatty acids are intermediate in fluidity between the more fluid *cis*-olefin and the less fluid *trans*-olefin as measured by differential scanning calorimetry (Silvius & McElhaney, 1979). Thus, accumulation of *cis*-olefins as a consequence of cyclopropanation inhibition may represent a strategy used by mycobacteria to increase cell-wall fluidity.

In addition to KasA, the expression of the mycobacterial Ag85 complex was also reduced in cells cultivated at 55 °C. In *M. tuberculosis*, Ag85A (FbpA), Ag85B (FbpB) and Ag85C2 (FbpC2) represent the three dominant exported proteins. By means of their fibronectin-binding activity, these proteins are supposed to be involved in the pathogenesis of tuberculosis. They have also recently been suggested to play a role in the final assembly of the mycobacterial cell wall by catalysing the transfer of mycolic acids to trehalose, to generate TDM and to the cell-wall AG (Belisle *et al.*, 1997; Kremer *et al.*, 2002b). The decreased expression of the Ag85 complex is consistent with the observation that cells cultivated at 55 °C produce less TDM than cells grown at 37 °C, as evidenced by two-dimensional TLC analysis of complex lipids. Altogether, our results suggest that the expression level of various enzymes involved in fatty acid/mycolic acid metabolism is regulated by temperature, thus participating in the environmental adaptation of the bacilli. Consistent with this notion, we have observed that the cytosolic enzyme fraction, which contains FAS-I and FAS-II, from cells grown at 55 °C presents a distinct electrophoretic profile different from that of the corresponding fraction from cells grown at 37 °C (data not shown). Whether the proteins that are down- or up-regulated at 55 °C belong to the FAS-II system remains to be investigated.

PIMs that are known to be precursors of LM and LAM have been proposed to recruit natural killer T cells which play a primary role in the granulomatous response (Apostolou *et al.*, 1999; Gilleron *et al.*, 2001). Moreover, a role for surface-exposed PIMs as *M. tuberculosis* adhesins that mediate attachment to non-phagocytic cells has also been established (Hoppe *et al.*, 1997). A recent study demonstrated a novel interaction between PIMs and Galectin-3, a galactoside-binding protein of macrophages (Beatty *et al.*, 2002), suggesting a role of these glycolipids in mycobacterial virulence. Our study shows that at elevated temperature both Ac₄PIM₆ and Ac₃PIM₆ synthesis is reduced, whereas expression of the other PIMs remains unchanged. This may be explained, at least partially, by a reduced mannosyltransferase activity as suggested by a decrease of GDP-[¹⁴C]Man incorporation into the PIM family using membranes from cells grown at elevated temperature. Although a clear function of PIM₆ has not yet been assigned, its

reduced expression may reflect significant changes in cell-wall fluidity and permeability. In addition, *in vitro* studies have shown that high temperature negatively affects PPM synthase activity responsible for C₅₀-P-Man synthesis. This glycolipid has previously been shown to be the mannose donor for LM/LAM biosynthesis (Gurcha *et al.*, 2002).

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