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Defective mycolic acid biosynthesis in a mutant of *Mycobacterium smegmatis*

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A mutant of *Mycobacterium smegmatis* defective in mycolic acid biosynthesis was isolated following chemical mutagenesis. Fatty acids were extracted from the mutant and subjected to structural analysis by thin-layer chromatography and high-performance liquid chromatography (HPLC) of both methyl and *p*-bromophenacyl ester derivatives. Thin-layer chromatography did not show the presence of any fatty acid of R_F comparable to that of standard methyl mycolate. The HPLC profile revealed a broad peak in the standard mycolic acid ester region. No characteristic peaks of mycolic acid esters comparable to the wild-type could be resolved. Mass spectral analysis of the HPLC-purified peak demonstrated the presence of shorter-chain fatty acids in the mutant. These data support the idea that the mutant accumulates precursors of mycolic acids and is incapable of carrying out the final conversion to mycolic acids of 60–90 carbon atoms.

Introduction

Mycolic acids are α-alkyl-β-hydroxy fatty acids unique to mycobacteria, nocardiae and corynebacteria (Gray *et al.*, 1982; Kaneda *et al.*, 1988; Minnikin & Goodfellow, 1980; Minnikin *et al.*, 1980; Qureshi *et al.*, 1978; Ratledge, 1982). They are major cell wall constituents. The number of carbon atoms which make up the mycolic acids varies from 60 to 90 in the genus *Mycobacterium* (Gray *et al.*, 1982; Kaneda *et al.*, 1988; Minnikin *et al.*, 1980). The basic structure is $R_1CH(OH)CH(R_2)COOH$, where R_2 is a linear alkane (C_{22}–C_{24}) and R_1 is a complex structure comprising about 60 carbon atoms and containing different functional groups including hydroxyl, methoxyl, carbonyl and carboxyl groups, cyclopropane rings, methyl branches and carbon double bonds (Steck *et al.*, 1978). Mycolic acids may play an important role in the pathogenicity of mycobacteria, since antituberculous drugs have been shown to inhibit mycolic acid biosynthesis (Takayama *et al.*, 1975), making it important to elucidate the pathway of mycolic acid biosynthesis.

Mycobacterium smegmatis, a rapidly growing cultivable mycobacterium, has been widely used to study mycobacterial metabolism (David, 1977; Matula *et al.*, 1971) and the biosynthesis of C_{16}–C_{32} fatty acids in *M. smegmatis* has been studied with cell-free extracts (Bloch & Vance, 1977). Lacave *et al.* (1990) have also established a cell-free system capable of synthesizing whole mycolic acid in *M. aurum*. The general structure of mycolic acids suggests a Claisen-like condensation reaction between two aliphatic chains. However, the details of the steps involved are still largely unknown (Takayama & Qureshi, 1984). We have isolated a mutant of *M. smegmatis* defective in synthesis of high-molecular-mass (C_{60}–C_{90}) fatty acids. This may be of considerable importance in studying mycolic acid biosynthesis. This is the first report of a mycobacterial mutant defective in mycolic acid synthesis.

Methods

Isolation of a penicillin-sensitive mutant. Mycobacterium smegmatis SN_2 was grown in nutrient broth. Cells grown to early exponential phase were suspended in 50 mM-potassium phosphate buffer, pH 6·5, containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), and incubated at 37 °C for 1 h. The cells were then harvested, washed to remove NTG under sterile conditions and serially diluted. The diluted suspensions were plated on solid nutrient broth (containing, l^{-1}: beef extract 10 g, peptone 10 g, NaCl 5 g; and 2%, w/v, agar) and incubated for 5 d. Mutants were selected for penicillin sensitivity by replica plating using different concentrations of penicillin G in the growth medium. A penicillin-sensitive mutant was finally picked using a single-colony selection procedure. Biochemical tests specific for *M. smegmatis* (Standard taxonomic tests; US Department of Health and Human Services, 1980) were carried out on the mutant and the wild-type. The arylsulphatase assay was carried out using *p*-nitrocatechol sulphate as a substrate. Catalase activity was measured by using hydrogen peroxide. For the nitrate reduction test, the mutant and the wild-type were grown in nitrate agar (containing 0·1% KNO_3) and the change in colour examined after addition of sulphanilic acid to the

medium. Iron uptake was studied by growing the organism in a medium containing 2·5% (w/v) ferric ammonium citrate and following the change in colour of the medium. Arabinogalactan, a characteristic cell wall component of mycobacteria, was isolated and analysed by gas–liquid chromatography after hydrolysis as described previously (Kundu *et al.*, 1990).

Isolation of fatty acids. These were isolated using a modification of the method described by Lanéelle *et al.* (1988). The cells were extracted with chloroform/methanol (2:1, v/v), washed with the same solvent, treated with tetrabutylammonium hydroxide overnight at 100 °C, then acidified with 6 M-HCl. Fatty acids were extracted from the reaction mixture using light petroleum (b.p. 40–60 °C).

Preparation of fatty acid methyl esters. The extracted fatty acids were methylated using ethereal diazomethane and dried. Methyl esters of fatty acids were spotted on to a silica gel GHL plate (Analtech) and developed with light petroleum/diethyl ether/acetic acid (9:1:0·2, by vol.). Spots on the plates were then visualized either by iodine vapour or by a dichromate and hot sulphuric acid spray.

Preparation of p-bromophenacyl esters of mycolic acids. These were prepared by the method of Butler & Kilburn (1988). Cells were extracted with 25% (w/v) KOH in ethanol/water (1:1, v/v) at 85 °C. The extract was then treated with chloroform, acidified with concentrated HCl, dried, treated with potassium bicarbonate and again dried. *p*-Bromophenacyl/crown ether mix (Pierce Chemical Co.) was added in 10-fold molar excess, heated at 85 °C for 20 min, filtered and the supernatant evaporated under vacuum.

Analysis of p-bromophenacyl esters of mycolic acids. High-performance liquid chromatography (HPLC) was performed on a Waters Associates model 6000A liquid chromatograph equipped with a variable-wavelength detector (model LC-85B; Perkin–Elmer) measuring absorbance at 254 nm. The mycolic acid *p*-bromophenacyl esters were applied to a 10 μm particle size Waters cartridge C_{18} reverse-phase column equilibrated with 91% methanol/9% chloroform. After injection of the sample, the gradient was changed linearly to 30% methanol/70% chloroform over a period of 65 min at a total flow rate of 2 ml min^{-1} (Butler *et al.*, 1986). Mass spectra were recorded on a ultrahigh resolution mass spectrometer, MS-50TC (Kratos Analytical Instruments) fitted with a D5-55 data acquisition system. Spectra were obtained at 70 eV ionization potential and 300 μA emission current.

Results

The colonies of the wild-type were rough and wrinkled whereas those of the penicillin-sensitive mutant were smooth and filamentous. Both the wild-type and the mutant satisfied the biochemical tests characteristic of *M. smegmatis*, i.e. positive for catalase (68 °C), amidase, nitrate reduction, iron uptake and presence of arabinogalactan, and negative for arylsulphatase. The mutant and the wild-type both contained arabinogalactan, a characteristic cell surface component of mycobacteria. Thin-layer chromatography of the methyl esters of fatty acids isolated from both the wild-type and the mutant cells after chloroform/methanol extraction was carried out using standard methyl mycolate from *M. smegmatis* (a generous gift from Dr K. Takayama). No methyl mycolate corresponding to the standard was obtained in the case of the mutant (Fig. 1).

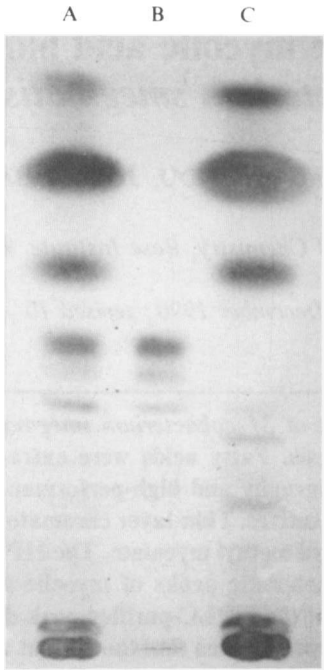

Fig. 1. Thin-layer chromatography of methyl mycolates of wild type *M. smegmatis* and its mutant: A, wild type; B, standard methyl mycolate; C, mutant.

Preparations of the *p*-bromophenacyl esters of mycolic acids from the mutant and the wild-type were dissolved in a minimum amount of chloroform/methanol (91:9, v/v) and injected into a reverse-phase column as described in Methods. A number of peaks, characteristic of the mycolates from *M. smegmatis* (Takayama & Qureshi, 1984), were obtained in the case of the wild-type cells. The peaks eluted between 36 and 48 min at a flow rate of 2 ml min^{-1} (Fig. 2*a*). For the mutant, however, only one broad peak was obtained (Fig. 2*b*). The fraction eluting between 36 and 48 min from the HPLC column was subjected to mass spectrometry. About 15–20 peaks in the M_r range 800–1200 were obtained in the case of the wild-type, while in the case of the mutant, a number of peaks of significantly lower M_r (600–850) were observed (data not shown).

Discussion

Earlier studies have indicated that mycolic acid biosynthesis might be the target of some antituberculous drugs (Takayama *et al.*, 1975). We have isolated a mutant of *M. smegmatis* defective in mycolic acid biosynthesis. The

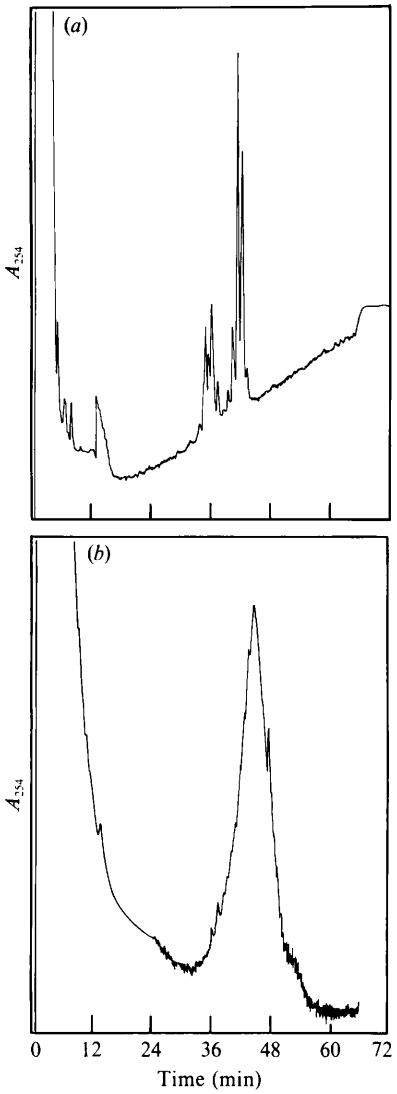

Fig. 2. HPLC profile of *p*-bromophenacyl esters of mycolic acid from (*a*) wild-type, (*b*) mutant.

mutant is taxonomically a mycobacterium on the basis of biochemical tests and by the presence of arabinogalactan, a typical cell wall polysaccharide of mycobacteria.

Thin-layer chromatography indicated the absence of methyl mycolates characteristic of *M. smegmatis*. Mass spectrometry of the mycolic acid *p*-bromophenacyl esters revealed the accumulation of shorter-chain fatty acids in the mutant, in contrast to the C_{60}–C_{90} fatty acids characteristic of the wild-type. These data suggest that the mutant is defective in mycolic acid biosynthesis.

Biosynthesis of mycolic acids in mycobacteria involves several steps. Elongation and Claisen-like condensation reactions between aliphatic chains occur with several intermediate reactions such as transacylation, carboxylation, reduction, dehydration and desaturation.

In *M. smegmatis* the newly synthesized mycolic acids have been reported to be transferred to final cell wall products via a mycolic acid exchange enzyme (Sathyamoorthy & Takayama, 1987). However, the biosynthetic machinery responsible for mycolic acid formation remains poorly understood. Qureshi *et al.* (1978) have identified two structural series of $C_{28:1}$–$C_{42:1}$ and $C_{55:2}$ meromycolic acids as possible precursors of monoenyl and dienyl mycolic acids respectively, since these acids are structurally related to the α-alkylhydroxyl group of the corresponding mycolic acid of *M. smegmatis* (Sathyamoorthy *et al.*, 1985). On the basis of the structures of different precursor fatty acids such as the C_{41}, C_{43}–C_{56} series, a possible pathway covering the precursor–product relationship between the C_{24}–C_{56} fatty acids and α-mycolic acids has been proposed by Takayama & Qureshi (1984). In this scheme C_{24-32} esters undergo a series of reactions involving elongation, desaturation and cyclopropane ring formation to yield the α-meromycolic acid esters (C_{52} or C_{54} esters). These products serve as the alkyl acceptor in the subsequent condensation reaction with another molecule of C_{24} or C_{26} fatty acid ester. However, present knowledge is not sufficient to establish a rigid relationship among the steps of the proposed pathway described above. The accumulation of shorter-chain fatty acids, possibly the precursors of mycolic acids, in the mutant suggests a block in the condensation between R_1–COOH (meromycolic acids) and the α-carbon of a C_{24}–C_{26} fatty acid. The purification, and subsequent characterization by mass spectrometry, of the individual components accumulating in the mutant will therefore be very useful in future studies involving detailed characterization of the intermediates present and their final conversion into mycolic acids. This will help to establish the different steps in the proposed pathway of mycolic acid biosynthesis.

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