Mycobactins and Exochelins of *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum* and Other Related Species

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Following iron-deficient growth, mycobactins and exochelins were isolated from 11 strains of *Mycobacterium tuberculosis* (including type strains of the virulent H37Rv and avirulent H37Ra organisms) as well as from *M. bovis* (one strain), *M. bovis* BCG (two strains), *M. africanum* (eight strains) and *M. xenopi* (one strain) but not from *M. microti* (one strain). The mycobactins from the tuberculosis group (*M. tuberculosis*, *M. bovis* and *M. africanum*) were identical and could each be resolved into four compounds by thin-layer chromatography (TLC) and into several fractions by high-performance liquid chromatography, supporting previous claims that this group is a single taxon. Exochelins were all chloroform-soluble and showed no species specificity; no single exochelin was recognized by TLC that had not been previously seen in *M. avium* or a related species.

INTRODUCTION

Like virtually all living organisms, pathogenic mycobacteria need iron. To meet the demand for iron, mycobacteria synthesize and utilize specific high-affinity iron-binding compounds which help them grow in the iron-restricted conditions of the host (Kochan *et al.*, 1971, Barclay & Ratledge, 1983, 1986*a, b*) and also participate in the uptake of iron across the thick lipid cell wall (Ratledge, 1982). Two types of iron-binding compound are produced (Ratledge, 1982): mycobactin, which is cell-associated, and exochelin, which is secreted into the extracellular environment. Mycobactins themselves differ structurally, and the types of mycobactin which are produced are determined by the species (Snow, 1970; Hall, 1986). *Mycobacterium tuberculosis* has been known for some time to produce one (Snow, 1965) or two mycobactins (White & Snow, 1968), but no comparison has been made between the mycobactins produced by virulent and avirulent tubercle bacilli, or between tubercle and closely related mycobacteria such as *M. bovis*, *M. africanum* and *M. microti*, which are usually grouped together as members of the 'tuberculosis' complex (Goodfellow & Wayne, 1982).

Nothing has been published about the exochelins of *M. tuberculosis* though it has been known for some time that the related *M. bovis* BCG produces a chloroform-soluble exochelin (Macham *et al.*, 1975). Chloroform-soluble exochelins seem to be important in the pathogenesis of other virulent mycobacteria such as *M. avium* and *M. paratuberculosis* (Barclay & Ratledge, 1983; Barclay *et al.*, 1985; Barclay & Ratledge, 1986*a, b*; Momotani *et al.*, 1986). In this report we have examined some of the properties of the mycobactins and exochelins from virulent and avirulent strains of *M. tuberculosis* and related organisms, and compared them to those of other pathogenic mycobacteria.

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Abbreviation: MAIS, *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum* group of organisms.

METHODS

Organisms and growth. The type of strains of *M. tuberculosis* used were NCTC 7417 (H37Ra) and NCTC 7416 (H37Rv). Freshly isolated strains of *M. tuberculosis* from P. A. Jenkins (PHLS Mycobacterium Reference Unit, University Hospital of Wales, Cardiff, UK) were strains 13398, 13661, 13965, 13968, 13969, 14039, 14043, 14045 and 14048. Isolates of *M. africanum* were from M. D. Yates (PHLS Regional Centre for Tuberculosis Bacteriology, Public Health Laboratory, Dulwich Hospital, London, UK) and included strains 40083, 40433, 40452, 40544, 41120, 41168, 41190 and 41396. Strains of *M. bovis* were NCTC 10772, and NCTC 5692 (BCG); *M. avium* strain M3 and *M. intracellulare* strain M12 were as described before (Barclay & Ratledge, 1983); *M. microti* (NCTC 8710), *M. triviale* (ATCC 23292) and *M. xenopi* (NCTC 10042) were also used. Strains were grown on an iron-deficient (20 ng Fe ml⁻¹) glycerol/asparagine/mineral salts medium (100 ml) which was prepared in iron-free glassware (Ratledge & Hall, 1971). Incubation was at 37 °C for 8 weeks without shaking. Iron-sufficient growth was on the same medium with 2 µg Fe ml⁻¹.

Mycobactin and exochelin isolation. Mycobactins were isolated by ethanol extraction of freshly harvested, moist mycobacterial cells (Snow, 1970). Exochelins were converted to their ferri-complexes and extracted into chloroform from cell-free culture filtrates (Macham *et al.*, 1975).

Thin-layer chromatography (TLC) of mycobactins and exochelins. The method used was that of Barclay & Ratledge (1983), with 250 µm thick silica gel 60 plates with concentrating zones (Merck). Mycobactins and exochelins were chromatographed with light petroleum (b.p. 60–80 °C)/n-butanol/ethyl acetate (2:3:3, by vol.). Exochelins were also separated with ethanol/light petroleum/ethyl acetate (1:4:6, by vol.).

High-performance liquid chromatography (HPLC). The method was adapted from that of Barclay *et al.* (1985) using a 20 × 0.25 cm column packed with Lichrosorb RP-18 with a solvent flow rate of 2 ml min⁻¹. For mycobactin and for exochelin separation, the mobile phase was initially 60% (v/v) methanol in water increasing to methanol only at a constant rate over 10 min. The mobile phase was thereafter maintained as 100% methanol.

Autoradiography. Exochelins in culture filtrates were labelled with ⁵⁵Fe, extracted into chloroform and separated by TLC, all as described by Barclay & Ratledge (1983). The solvents were completely removed from the TLC plates by air-drying. Autoradiography of the plates was then done with Kodak X-Omat RB X-ray film (18 × 24 cm) with an exposure for 7 d at room temperature.

RESULTS AND DISCUSSION

Production of mycobactin and exochelin

Twenty-three strains of *M. tuberculosis* and related species of mycobacteria were examined for the production of mycobactin and exochelin, after iron-deficient growth. All eleven strains of *M. tuberculosis*, three of *M. bovis*, including two of BCG, and eight of *M. africanum* produced both mycobactin and exochelin but *M. microti* (one strain only) appeared to produce neither. Production of mycobactin and exochelin reached a maximum after 6–8 weeks with the amounts produced varying according to the strain used (Table 1). The ability of the tuberculosis group to produce exochelin decreased with repeated subculture in laboratory growth media, whilst mycobactin production increased. This phenomenon has also been observed for members of the MAIS group, i.e. *M. avium*, *M. intracellulare*, *M. scrofulaceum* and *M. paratuberculosis* (Barclay & Ratledge, 1983).

Mycobactins

Mycobacteria may be differentiated by the mobility of their mycobactins in TLC systems and this is a useful tool in the classification of most mycobacteria (Ratledge, 1987). Early reports suggested that *M. tuberculosis* probably produced two mycobactins (White & Snow, 1968), but improved TLC techniques now show that there are four main components (Table 2). Furthermore, the separation pattern with respect to the *R_F* values of the four components was consistent for the mycobactins from the tubercle and related bacilli (*M. bovis*, *M. bovis* BCG and *M. africanum*) but differed from that of other mycobacterial species (Table 2). The virulent (e.g. H37Rv) and avirulent (H37Ra) strains of tubercle bacilli had identical separation patterns for their mycobactins. Other TLC solvent systems, which had been used successfully to differentiate the mycobactins of other species (Barclay & Ratledge, 1983; Barclay *et al.*, 1985), failed to differentiate the mycobacteria of the tuberculosis group (results not shown).

Mycobactins can also be differentiated by the nature of their long alkyl chains using HPLC. This separation differs from that of the TLC method, which relies on variations in the polar

Table 1. *Production of mycobactins and chloroform-soluble exochelins by organisms of the tuberculosis group after 8 weeks at 37 °C*

Results are means of four experiments.

Mycobacterium species and strain no.*	Mycobactin production [$\mu\text{g (g cell dry wt)}^{-1}$]		Exochelin production [$\mu\text{g (g cell dry wt)}^{-1}$]	
	Iron- sufficient growth	Iron- deficient growth	Iron- sufficient growth	Iron- deficient growth
<i>M. tuberculosis</i>				
NCTC H37Ra	1492	3852	531	1074
NCTC H37Rv	ND	1455	ND	1032
13398	ND	1324	ND	938
13661	ND	2036	ND	1282
13965	794	1836	64	1118
13968	ND	4302	ND	1142
13969	274	4222	488	604
14039	454	920	1032	3402
14043	226	700	1478	2700
14045	554	670	20	1026
14048	0	1200	398	562
<i>M. africanum</i>				
40083	ND	55	ND	636
40433	ND	1135	ND	384
40452	ND	956	ND	126
40544	ND	1554	ND	1128
41168	ND	0	ND	796
41190	ND	0	ND	662
41396	ND	1327	ND	0
<i>M. bovis</i> BCG	298	453	108	411
<i>M. microti</i>	0	0	0	0
<i>M. xenopi</i>	ND	4166	ND	102
<i>M. triviale</i>	ND	132	ND	403

ND, Not determined.

* For origin of strains see Methods.

Table 2. *Identification of mycobactins by TLC*

Source of mycobactin	No. of strains examined	R_F in given solvent system*			
Avirulent <i>M. tuberculosis</i> (H37Ra)	1	0.45	0.48	0.60	0.64
Relative percentage (w/w)†		(15%)	(22%)	(35%)	(28%)
Virulent <i>M. tuberculosis</i> (H37Rv)	10	0.45	0.48	0.60	0.64
<i>M. bovis</i>	1	0.45	0.48	0.60	0.64
<i>M. bovis</i> BCG	2	0.45	0.48	0.60	0.64
<i>M. africanum</i>	8	0.45	0.48	0.60	0.64
<i>M. xenopi</i>	1		0.81		
<i>M. avium</i>	‡		0.89		

* Light petroleum/butanol/ethyl acetate (2:3:3, by vol.).

† Calculated by elution with ethanol and determination of absorbance at 450 nm.

‡ From Barclay *et al.* (1985).

nucleus of the molecule (Barclay *et al.*, 1985), and consequently the elution profiles given by the two processes bear no relationship to each other. HPLC has been used successfully to resolve mycobactins of slow-growing and rapidly growing mycobacteria (Barclay *et al.*, 1985; Hall & Ratledge, 1984, 1985*a, b*), but had not been applied to those from tubercle bacilli. The elution profile for *M. tuberculosis* H37Ra (Fig. 1) was typical in its major features of all the tubercle and

Fig. 1

Fig. 1. Reverse-phase HPLC of mycobactins from *M. tuberculosis* H37Ra on a Lichrosorb RP 18 column with a gradient of 60% (v/v) methanol in water rising to 100% methanol in 10 min. Elution times (in seconds) of the major peaks are indicated.

Fig. 2

Fig. 2. Autoradiograph after TLC (light petroleum/butanol/ethyl acetate; 2:3:3 by vol.) of exochelins (extracellular ^{55}Fe -binding compounds) from *M. tuberculosis* strain 13965 (lane 1), 13969 (lane 2), 13968 (lane 3), H37Ra (lane 4) and H37Rv (lane 5).

related bacilli described in Table 1, and was distinct from that of other unrelated mycobacteria (Barclay *et al.*, 1985; Hall & Ratledge, 1984, 1985*a, b*). Unlike the mycobactins of the MAIS group (Barclay *et al.*, 1985; Barclay & Ratledge, 1983, 1986*a, b*), the mycobactins of the tuberculosis group could not be used to differentiate the individual members of the group by HPLC.

Exochelins

Exochelins in the culture filtrates of the organisms described in Table 1 were labelled with ^{55}Fe and extracted with chloroform as before (Barclay & Ratledge, 1983), separated by TLC and then autoradiographed. Each sample separated into a number of bands of radioactivity (Fig. 2) that corresponded to the orange (exochelin) bands visible on the TLC plate. The patterns were similar in complexity to those previously recorded for the exochelins of the MAIS and *M. paratuberculosis* groups (Barclay & Ratledge, 1983). No exochelin components from the tubercle bacilli were found that had not been previously seen with the MAIS and *M. paratuberculosis* groups. Furthermore, no radiolabelled components were found remaining in the culture filtrates after chloroform extraction, which would suggest that no water-soluble exochelin was produced. Thus, a common and distinct property of most of the slow-growing pathogenic mycobacteria is the ability to produce a number of related chloroform-soluble exochelins.

The ferri-exochelins from the mycobacterial strains shown in Table 1, as well as those of *M. avium* M3, *M. intracellulare* M12 and *M. paratuberculosis* strain 18, were further examined by

Fig. 3. Reverse-phase HPLC of exochelins from *M. tuberculosis* H37Ra on a Lichrosorb RP 18 column with a gradient of 60% (v/v) methanol in water rising to 100% methanol in 10 min.

HPLC, a single example of which is shown in Fig. 3. Other solvent systems incorporating methanol, acetonitrile, propan-1-ol and water under various gradient systems were examined but were not as effective as the methanol/water gradient system (results not shown). All exochelins exhibited differing elution patterns, similar in complexity to that shown in Fig. 3, but no differences occurred which could be construed as the result of species differentiation. Thus, the exochelins are unlikely to be of use as chemotaxonomic characters in differentiating mycobacteria but the data obtained by both TLC and HPLC analyses confirms the close-relatedness of this group of iron-chelating materials from the tubercle, MAIS and *M. paratuberculosis* groups of mycobacteria.

One of the aims of studying iron-uptake mechanisms of pathogenic mycobacteria is to pinpoint new sites for antimycobacterial drugs. The finding that the 'tuberculosis' group of organisms produce extracellular iron-binding compounds that are unique to them and other slow-growing mycobacterial pathogens such as the MAIS group, including *M. paratuberculosis*, is therefore of great interest. This is particularly so given that exochelins are produced mostly by freshly isolated strains than by laboratory-subcultured ones and suggests that exochelins are the preferred means of acquiring iron in the host. These findings make it likely that an understanding of the iron-uptake mechanisms of any one of the slow-growing pathogens will result in an understanding of them all. Furthermore, the similarity in the exochelins produced by the mycobacteria of the 'tuberculosis' group to those produced by the MAIS group is of taxonomic interest, and suggests that these pathogens may have evolved by a pathway distinct from that of the saprophytes.

We have shown that like the other major species the tuberculosis group, with the exception of *M. microti*, produce characteristic mycobactins. Unlike the mycobactins from the MAIS group, those from the tuberculosis group could not be differentiated by HPLC which further reinforces the view that these organisms belong to a single taxon (Goodfellow & Wayne, 1982). However, as the attenuated strains, such as *M. tuberculosis* H37Ra and *M. bovis* BCG, produce the same mycobactins and exochelins as their more virulent counterparts, it is clear that although an

effective iron-uptake system is essential to a pathogen, siderophore production in itself is not a sole virulence determinant of tubercle bacilli. Nonetheless, inhibition of iron uptake into these bacilli would be potentially useful for the inhibition of their growth *in vivo*.

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