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Isolation, Identification, and Structural Analysis of the Mycobactins of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, and *Mycobacterium paratuberculosis*

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Methods were devised to purify the cell-associated, iron-binding compounds known as mycobactins from the closely related species *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* (i.e., the MAIS complex of organisms). The mycobactins from these three species showed a structure that is common to the mycobactins from all the mycobacteria examined to date. However, these mycobactins were unique in that they had more than one alkyl chain. The *M. scrofulaceum* mycobactins differed from other MAIS mycobactins by a shift in the position of the double bond in the R1 alkyl chain. Traces of other mycobactin types were observed in ethanol extracts of the three species, and examination of the chromatographic properties of these mycobactins showed that each species produced five mycobactin types. Each mycobactin could be subdivided further by the length of its R1 alkyl chain. No differences in the production of these novel mycobactins were observed among species. Mycobactins from three strains of *Mycobacterium paratuberculosis* and two wood pigeon strains of *Mycobacterium avium* which had lost their original growth requirements for mycobactin after repeated subculturing in laboratory growth media were examined by thin-layer chromatography and high-pressure liquid chromatography. Each organism produced a mycobactin with similar chromatographic properties to those synthesized by MAIS organisms. *M. paratuberculosis* NADC 18 produced at least two components in our laboratory, and nuclear magnetic resonance analysis of the major component showed this mycobactin to be identical to that produced by *M. intracellulare* M12. However, a sample of mycobactin J isolated by Merkal and McCullough (Curr. Microbiol. 7:333–335, 1982) from *M. paratuberculosis* NADC 18 was different from our isolates and appeared to correspond to a minor mycobactin component we had seen by thin-layer chromatography. No reason for this difference could be evinced. Our findings indicate that there is a close taxonomic relationship between *M. paratuberculosis* and the MAIS complex.

The mycobactins are a group of closely related cell-associated, iron-binding compounds that are produced by mycobacteria under iron-limiting conditions. The general structure of the mycobactins (Fig. 1) was determined by Snow (18). The iron-chelating nucleus is rendered lipid soluble by the presence of an alkyl chain, of variable length, positioned at either position R1 or R4. As different mycobacteria produce distinctive mycobactins, by virtue of the fact that there are different substituents at the positions R1 to R5, Snow (19) proposed that mycobactins could be useful in the identification and taxonomy of the mycobacteria. Greatbanks and Bedford (3) advanced a system for identifying known mycobactins by their nuclear magnetic resonance (NMR) spectrum alone. However, results of subsequent work in our laboratory (1, 5, 16) have shown that the distinctiveness of a mycobactin can be determined chromatographically, although the structural elucidation of the molecule still requires NMR spectroscopy.

Mycobacterium avium, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* taxonomically are closely related organisms. They have been defined as the MAIS complex of organisms which grow slowly and, when compared with the fast-growing mycobacteria such as *Mycobacterium smegmatis*, produce relatively little mycobactin (1). Although the structures of the mycobactins produced by the MAIS organisms have not been determined in our previous investigation, it is known that the mycobactins from the MAIS organisms all have equivalent chromatographic properties on thin-layer chromatographic (TLC) systems that are distinct from those of the other mycobacteria (1, 8). In this study we were concerned with determining the structures of the mycobactins produced by each member of the MAIS complex in view of the putative equivalence of these organisms.

Mycobacterium paratuberculosis, which is the etiological agent of Johne's disease in cattle, shares with the strains of *M. avium* from wood pigeons the inability for freshly isolated strains to be cultivated in vitro without the addition of an iron-chelating siderophore. This siderophore may be either a mycobactin or an exochelin (1). Exochelins are water-soluble, extracellular siderophores of the mycobacteria (13) and represent the probable means by which iron is acquired for growth in vitro and in vivo.

Some laboratory-cultured strains of *M. paratuberculosis* and of *M. avium* from wood pigeons spontaneously lose their dependency on mycobactin (7, 9), and this coincides with the production of mycobactin by these organisms (1, 9, 10, 11). The inability to produce mycobactin therefore would appear to be a phenotypic process rather than one that is due to the loss of genetic information. However, although *M. paratuberculosis* is regarded by taxonomists as being closely related to *M. avium* (2, 21), the structure of this mycobactin termed mycobactin J has been proved to be different from the ones we determined for the mycobactins from *M. avium* and *M. intracellulare*. Therefore, we have reexamined the

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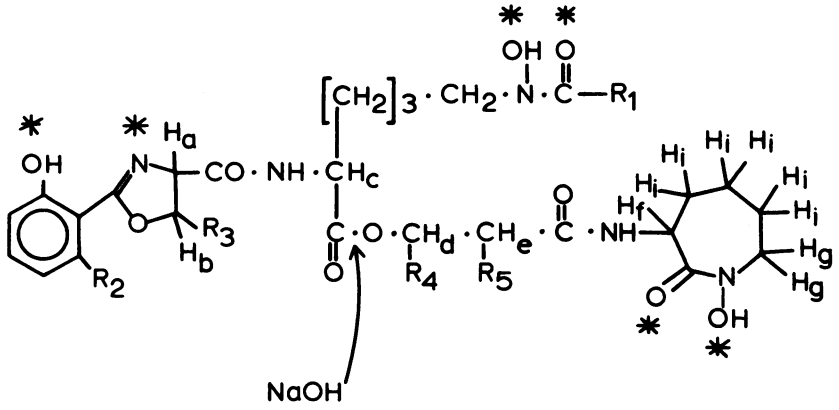


FIG. 1. General structure of the mycobactins. Asterisks indicate Fe binding sites.

mycobactin from *M. paratuberculosis* to resolve this apparent anomaly.

MATERIALS AND METHODS

Organisms and growth. The *M. avium* strains studied were M1, M3, 5(W), and 8(W). *M. intracellulare* strains studied were M12, M18, M24, M36, 15769, and 17573. The *M. scrofulaceum* strain was NCTC 1083. All of the strains listed above were as described previously (1). The cultures were grown on an iron-deficient (20 ng of Fe per ml) glycerol-asparagine-mineral salts medium (100 ml) which was prepared in iron-free glassware as described previously (15). Incubation was at 42°C for 8 weeks without shaking. *M. paratuberculosis* NADC 18 was kindly donated by R. S. Merkal, U.S. Department of Agriculture, North Central Regional National Animal Disease Center, Ames, Iowa. *M. paratuberculosis* 465-BC(W) and C57/2 and the *M. avium* WP 1/77 and WP 7/74 from wood pigeons were as described previously (1), but they subsequently lost their dependence on mycobactin after they were repeatedly subcultured on laboratory media. All of the *M. paratuberculosis* strains were maintained on Stuart's medium (20) modified by the inclusion of 30 μg of mycobactin S per ml and solidified by the inclusion of 2% (wt/vol) agar. The liquid culture medium (100 ml) was either the iron-deficient medium described above or the medium described by Merkal and McCullough (10) which was similar to the liquid medium described above, but it also contained sodium citrate (0.9 g) and glucose (10 g). *M. paratuberculosis* NADC 18 was incubated for 3 weeks at 38°C (10) without the addition of any mycobactin. All other *M. paratuberculosis* strains were incubated for 10 weeks at 37°C (2).

Mycobactin isolation. The mycobactins were isolated by the ethanol extraction of mycobacterial cells by the method of Snow (19) or that of Merkal and McCullough (10). A sample of mycobactin J, which had been isolated from *M. paratuberculosis* NADC 18, was kindly donated to us for this work by R. S. Merkal.

Thin-layer chromatography. TLC was carried out as described by Barclay and Ratledge (1) with 250-μm-thick silica gel 60 TLC plates with concentrating zones (E. Merck AG, Darmstadt, Federal Republic of Germany). Solvent system A consisted of petroleum ether (60 to 80°C)–*n*-butanol–ethyl acetate (2:3:3 [vol/vol/vol]). Solvent system B was ethanol-petroleum ether-ethyl acetate (1:4:6 [vol/vol/vol]), and solvent system C was petroleum ether-ethyl acetate-methanol (49:49:2 [vol/vol/vol]).

Purification procedure. The procedure adopted for purification differed from those of previously published methods (13) because of the different nature of the mycobactins (see below). The mycobactin (50 mg of an ethanol cell extract) was applied to a column (80 by 2.5 cm) of Sephadex LH-20 and eluted with ethanol. The main red band of mycobactin was collected, the solvent was evaporated, and the mycobactin was suspended in petroleum ether (60 to 80°C). When this was applied to a column (20 by 2.5 cm) of silica gel 60, the mycobactin was retained at the top of the column. The column was washed with 1 liter of petroleum ether before the mycobactin was eluted with methanol. The mycobactin was collected, and the solvent was evaporated to dryness, suspended in petroleum ether, and applied to silica gel 60 TLC plate (approximately 5 mg of sample per plate). The plate was developed with solvent system A. Under UV light (260 nm) the dark, absorbing mycobactin was seen to have ben partially separated from other, flourescent components. The mycobactins were extracted from the plate with chloroform, filtered, and applied to a second TLC plate which was developed with solvent system B. The mycobactins were extracted with chloroform, filtered, and suspended in 3 ml of chloroform. Iron was removed from the mycobactin by gently shaking the chloroform suspension with 1 ml of methanol plus 1 ml of 6 M HCl. The resulting colorless chloroform solution, which contained about 10 mg of desferri-mycobactin, was evaporated in iron-free glassware under a stream of dinitrogen gas. The mycobactin was stored in vacuo over P_2O_5.

Mycobactin hydrolysis. The method used for mycobactin hydrolysis was essentially that of Snow (18), in which the cleaned desferri-mycobactin was dissolved in 0.2 ml of methanol and incubated in 1 ml of 1 M NaOH for 1 h at 20°C. Hydrolysis was stopped by neutralization with 1 ml of 1 M HCl. The hydrolysate was filtered through Whatman no. 1 filter paper and then extracted three times with 3 ml of diethyl ether. The ether phases, which contained mycobactic acid, were pooled, washed with distilled water, and dried over $MgSO_4$. The aqueous layer from the ether extraction, which contained the cobactin, was evaporated to dryness and then extracted five times with 20 ml of boiling acetone. The acetone was filtered and evaporated to dryness, leaving the cobactin as a residue.

NMR spectroscopy at 400 MHz. The desferri-mycobactins were analyzed on a Bruker WH-400 instrument in the

Chemistry Department, Sheffield University, Sheffield, United Kingdom. Spectra were obtained in $CDCl_3$ at 400.134 MHz with a 5-μs pulse, an acquisition time of 3.28 s, and a sweep width of 500 Hz with the fid collected into 32 K.

High-pressure liquid chromatography. Analysis involved a Spectra Physics 8000 high-pressure liquid chromatographic (HPLC) system with spectrophotometric detection of the eluted sample at 450 nm. The column (20 by 0.25 cm) was packed with Lichrosorb RP-8. All solvents, including water, were of HPLC grade and were degassed with helium prior to and during each chromatographic run. The mobile phase initially was 80% (vol/vol) methanol in water and was raised to 100% (vol/vol) methanol at a constant rate in 30 min. Thereafter, the mobile phase was maintained as 100% methanol. The flow rate was constant at 2 ml/min. The column pressure was approximately 100 atm. Relative proportions of the eluted components were calculated by the internal data-handling section of the HPLC system after they were read by the spectrophotometer. Retention times were calculated in seconds.

RESULTS

Purification and analysis. Previous chromatographic methods for the purification of the mycobactins from *M. smegmatis*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium terrae*, *Mycobacterium marinum*, *Mycobacterium aurum*, and others (13, 18) yielded highly purified compounds that were suitable for examination by 60-MHz proton NMR spectroscopy (2). When these purification methods were used in an attempt to isolate MAIS mycobactins for NMR analysis the products were only about 80% pure (based on a 1% E_{450} of about 37.5; see below). This poor yield was considered to be the result of fundamental differences in the composition of the mycobactins which were reflected in the different chromatographic properties of MAIS mycobactins from those of other mycobacteria (1, 8). A new purification method for MAIS mycobactins was devised accordingly (see above).

Proton NMR spectroscopy of the mycobactins, purified by this new method, at 400 MHz showed the absence of nonmycobactin molecules. However, after the proton resonance peaks were designated by the methods of Greatbanks and Bedford (2), it was apparent that at least two mycobactins were present in each sample. With *M. scrofulaceum* the two mycobactins were present at a ratio of about 2:1, whereas the mycobactins from the other species consisted predominantly of one mycobactin type, with less than 5% of the other mycobactin types being present. In all of the mycobactins examined, the minor component had proton resonance peaks that were broader than those of the major components. Because iron binding is the most likely cause of peak broadening, this indicates that the minor component probably has a greater affinity for iron than does the major component.

By using a less polar solvent system (C) we were able to separate each mycobactin component from *M. avium* M3 and M36, *M. intracellulare* M12, and *M. scrofulaceum* by TLC. Results of TLC showed that the same two mycobactin components (R_f values of 0.37 and 0.26) were present in the crude ethanol cell extracts (in ratios of about 2:1), but small amounts of three additional components (R_f values of 0.20, 0.14, and 0.08) were also there. Thus, organisms of the MAIS complex produce two main mycobactins and three further minor components. The 1% E_{450}s of the *M. intracellulare* M12 mycobactin in methanol were 37.4 and 38.9 for the major and minor components, respectively, which were lower than the value of 42 reported previously for other mycobactins (18).

HPLC analysis of the MAIS mycobactins. The ferrimycobactins from *M. avium* M1, M3, 5(W), 8(W), WP 1/77, and WP 7/74; *M. intracellulare* 15769, 17573, M12, M18, M24, and M36; *M. scrofulaceum*; and *M. paratuberculosis* C57/2, 465-BC(W), and NADC 18 were examined by HPLC as described above. The elution profile for *M. intracellulare* M12, which was typical of all the mycobactins produced by the *M. avium* and *M. intracellulare* strains examined, is shown in Fig. 2. The most prominent peaks were designated 1 to 9. The elution profile for *M. scrofulaceum* (Fig. 3) was similar to that for *M. intracellulare*, but the peak retention times were increased. A second difference between *M. scrofulaceum* and other MAIS organisms was that the abundance of peaks 7 and 8 was reversed in the sample from *M. scrofulaceum*.

When peaks 5 through 9 were separately collected after HPLC of each mycobactin and then examined by TLC with solvent system C, no differences were seen between the R_f values of any of these peaks. The TLC pattern observed was exactly the same as that seen with the original ferri-

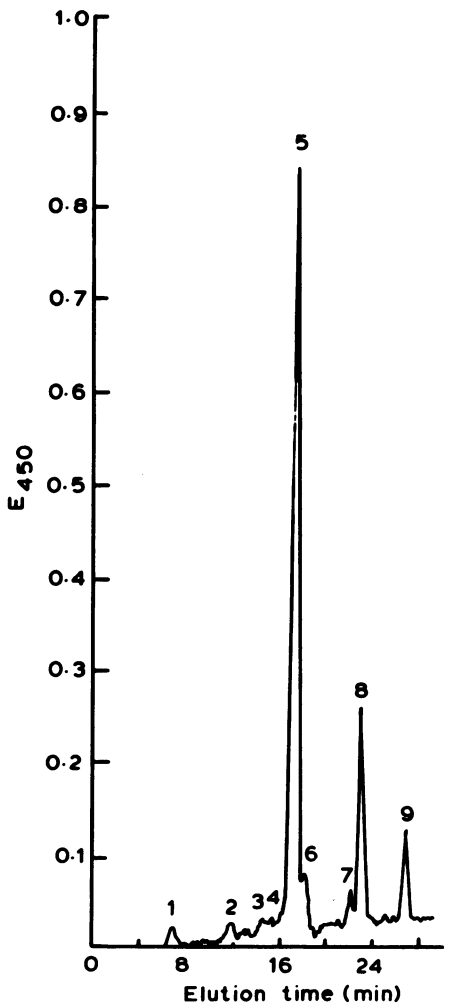


FIG. 2. Reverse phase HPLC of *M. intracellulare* M12 mycobactins on a Lichrosorb RP-8 column with a gradient of 80% (vol/vol) methanol in water, rising to 100% methanol in 30 min. Numbers on the peaks correspond to nine identifiable fractions.

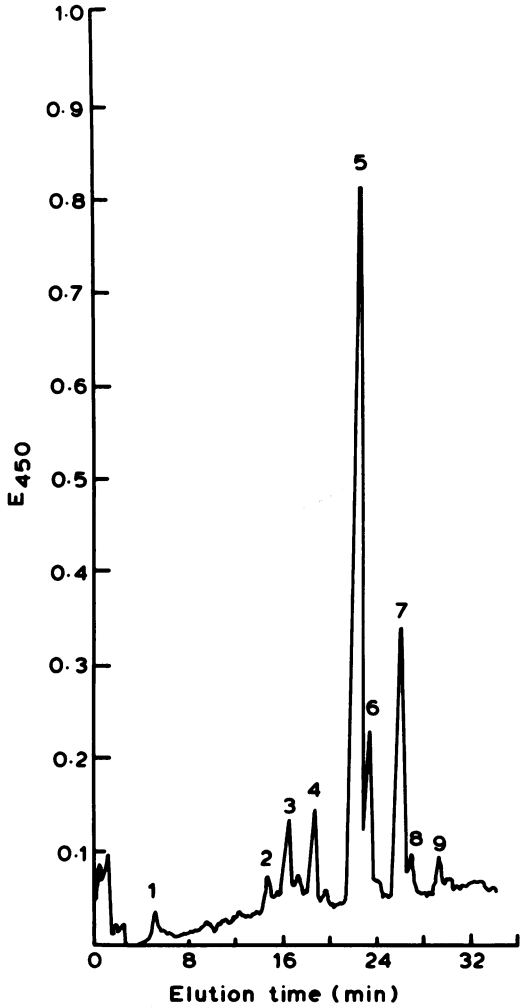


FIG. 3. Reverse phase HPLC of *M. scrofulaceum* mycobactins on a Lichrosorb RP-8 column with a gradient of 80% (vol/vol) methanol in water, rising to 100% methanol in 30 min. Numbers on the peaks correspond to those of the *M. intracellulare* mycobactins.

mycobactin used as a control (see above). Therefore, it was concluded that TLC and HPLC separated the mycobactins by different sets of structural properties.

NMR analysis of the MAIS mycobactins. As *M. intracellulare* M12 produced about 6 mg of mycobactin per g (dry weight) of cell compared with an equivalent value of 3 to 5 mg for the other MAIS organisms, this mycobactin was used as the principal model for the purification and analysis of the MAIS mycobactins. The 400-MHz proton NMR spectrum of this purified mycobactin is shown in Fig. 4, and the main features of the spectra for other MAIS mycobactins are summarized in Table 1.

The resonances common to all the mycobactins were as follows: the protons in the benzene ring at 6.90 to 7.78 ppm, a hydroxyl group at 6.44 ppm, protons in the aliphatic chains at 1.24 ppm, and protons on the R1-terminal methyl groups at 0.82 to 0.90 ppm. For the major component from each organism the triplet at 0.86 ppm had an area corresponding to that of two essentially identical methyl groups. One of these methyl groups belonged to the unsaturated alkyl chain of the R1 group, whereas the other was identified as part of group R4. The nature of the R4 group, however, was different from that recovered from all other mycobactins (3). The mycobactin from *M. intracellulare* M12 differed from its equivalents in the other MAIS organisms by having an R3 group that was a methyl instead of a proton. The mycobactins from *M. scrofulaceum* also differed from those of *M. avium* and *M. intracellulare* in the position of the double bond in the R1 alkyl chain. This unsaturated bond was not at the [α,β]-position, as with the other organisms, but was located further along the alkyl chain; the exact location of this double bond was not deduced.

NMR analysis of the cobactins. In an attempt to determine the nature of the R4 alkyl chain, the mycobactin from *M. intracellulare* M12 was hydrolyzed with alkali under iron-free conditions (see above) to give cobactin and mycobactic acid (which contains the aromatic residue; Fig. 1). The cobactin part of the molecule, which contains the R4 group, was isolated and examined by 400-MHz NMR spectroscopy. The resonance peaks on the spectrum were sharper than those obtained with the complete mycobactin molecule because cobactin has no significant iron-binding capacity. The spectrum was consistent with the hydroxy acid expected from M12 mycobactin, and none of the protons of the mycobactic acid part of the mycobactin molecule were observed. Only one terminal methyl group was present, at 0.96 ppm, and the CH_2 groups of the R4 alkyl chain were apparent at 1.45, 1.64, and 2.0 ppm. There was no evidence of unsaturation or branching within the R4 chain; however, the length of the R4 chain could not be accurately determined because the peaks overlapped those from the three CH_2 groups in the seven-membered ring. From the area of the peak at 1.64 ppm, however, the best estimated structure for R4 is a 10-carbon straight alkyl chain.

NMR analysis of the minor mycobactin components. Although all the TLC systems yielded two major mycobactin components, it was only with the mycobactins from *M. scrofulaceum* that a structural analysis of the minor component was possible from the original NMR spectrum (Table 1). Unlike the mycobactins produced by the other MAIS organisms, the peaks of the benzene ring protons of the second *M. scrofulaceum* component were moved slightly upfield. In addition there was no evidence of an alkyl chain at the R4 position, and this group was deduced to be CH_3. Like the major component of this sytem, the —CH=CH— bond in the R1 alkyl chain was not adjacent to the main part of the mycobactin molecule. When compared by HPLC with the other MAIS mycobactins, the differences in the retention times of the *M. scrofulaceum* mycobactins were probably accounted for by the position of the double bond.

TLC of *M. paratuberculosis* mycobactins. Mycobactin J, which has been isolated from *M. paratuberculosis* NADC 18 by R. S. Merkal, together with those mycobactins isolated in our own laboratory by the methods of Snow (19) or Merkal and McCullough (10) from *M. avium* WP 1/77 and WP 7/74 and from *M. paratuberculosis* C57/2, 465-BC(W), and NADC 18 itself, were examined by TLC. With the exception of mycobactin J (Table 2), all the mycobactins had the same R_f value (0.89) in the solvent system used as that found previously for *M. avium* and related species (1). The mycobactin isolated in our laboratory from *M. paratuberculosis* NADC 18 grown as closely as possible under the conditions described by Merkal and McCullough (10), separated into two components on TLC. The main component, which accounted for about 90% of the mycobactin, migrated like the *M. paratuberculosis* and MAIS mycobactins described above, whereas the second component had an R_f value of 0.42 (Table 1). However, the mycobactin supplied by R. S. Merkal separated into four

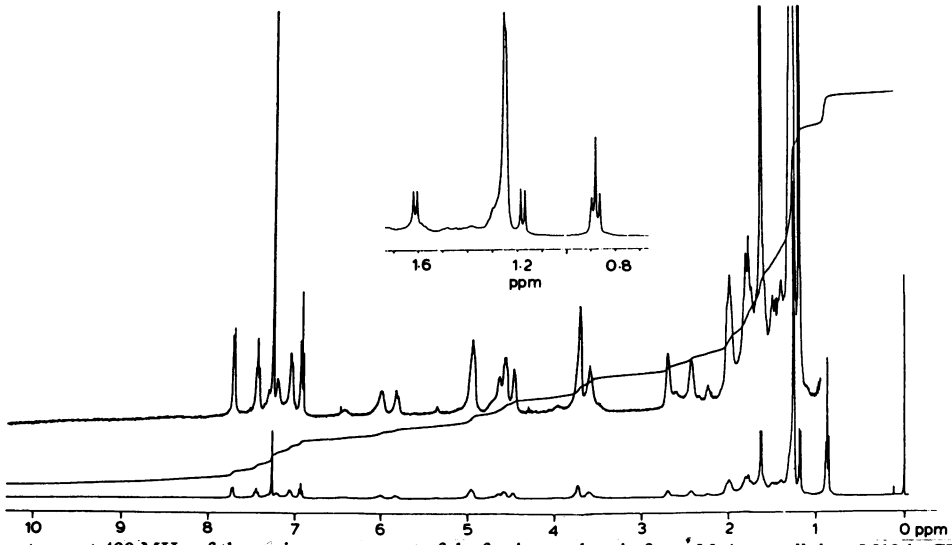


FIG. 4. NMR spectrum at 400 MHz of the major component of desferri-mycobactin from *M. intracellulare* M12 in CDC1. Three levels of amplification and an integration are shown. The triplet at 0.86 ppm corresponds to the terminal methyl groups of the two alkyl chains of the mycobactin molecules.

components. The main component, which accounted for about 80% (dry weight) of the mycobactin, had an R_f value of 0.42, whereas the minor components had R_f values of 0.60, 0.72, and 0.89, respectively. No differences in the mobilities of the mycobactins were observed when different extraction methods were used to obtain the mycobactins from harvested bacteria. Such extraction methods included that described by Merkal and McCullough (10).

HPLC of *M. paratuberculosis* mycobactins. Reverse phase HPLC of the *M. paratuberculosis* strains examined by TLC resulted in a separation pattern similar to that of *M. paratuberculsois* NADC 18 (Fig. 5). This separation is comparable to the one described for the same organisms by Merkal et al. (11), although we used a C18-bonded silica instead of a C8-bonded silica in the separation column. The pattern obtained was also similar to that described above for the MAIS mycobactins. Resolution of the individual mycobactins was probably by virtue of the alkyl chain length of the R1 group of the molecule, as indicated above. The *M. paratuberculosis* mycobactin was slightly different from the *M. avium* type in that peak number 7 (Fig. 2 and 5) was about 1.4 times the area of peak 8, whereas in *M. avium* it was only 0.16 times the area of peak 8. No differences in elution profile were observed among the mycobactins extracted by the two different methods described above.

Taken together, the results from the TLC and HPLC analyses suggest that the mycobactins produced by the *M. paratuberculosis* that we grew are similar to those of *M. avium* (1; see above). However, our results did not agree with those of Merkal and McCullough (8, 10), who found that

TABLE 1. Identification of the peaks in the NMR spectra of MAIS mycobactins

Source of mycobactin	Identities of the following mycobactins:										
	R1			R2		R3		R4		R5	
	Group	ppm	—CH=CH— position	Group	ppm	Group	ppm	Group	ppm	Group	ppm
M. intracellulare M12	Unsaturated alkyl chain	5.82 + 6.00	[α,β]	H	7.72	CH_3	1.62	Saturated alkyl chain	0.86 + 1.25 + 1.98	CH_3	1.17
M. avium M3	Unsaturated alkyl chain	5.84 + 6.00	[α,β]	H	7.73	H	4.58	Saturated alkyl chain	0.86 + 1.26 + 1.96	CH_3	1.17
M. avium M36	Unsaturated alkyl chain	5.85 + 6.00	[α,β]	H	7.76	H	4.57	Saturated alkyl chain	0.88 + 1.25 + 1.99	CH_3	1.17
M. scrofulaceum											
Major component	Unsaturated alkyl chain	5.64	Not [α,β]	H	7.76	H	4.5	Saturated alkyl chain	0.88 + 1.24 + 1.98	CH_3	1.17
Second component	Unsaturated alkyl chain	5.64	Not [α,β]	H	7.76	H	4.5	CH_3	1.32	CH_3	1.11
M. paratuberculosis 'J'	Unsaturated alkyl chain	5.85	[α,β]	H	7.73	CH_3	1.61	Saturated alkyl chain	0.86 + 1.22 + 1.98	CH_3	1.16

TABLE 2. TLC of *M. paratuberculosis* and wood-pigeon *M. avium* mycobactins with solvent system A

Component (R_f)	Approx % (dry wt) mycobactin in:					
	M. paratuberculosis				*M. avium*	
	J[a]	"J"[b]	C57/2	465-BC(W)	WP1/77	WP7/74
0.89	10	90	100	100	100	100
0.72	5	0	0	0	0	0
0.60	5	0	0	0	0	0
0.42	80	10	0	0	0	0

[a] As kindly supplied by R. S. Merkal, being isolated from *M. paratuberculosis* NADC 18.

[b] Mycobactin isolated after growth of *M. paratuberculosis* NADC 18 in our laboratories.

mycobactin J is the major mycobactin component produced by *M. paratuberculosis* NADC 18. These findings were confirmed when a second culture of *M. paratuberculosis* NADC 18, also obtained from R. S. Merkal, was examined by us.

An explanation for the difference in the findings between the two groups could be that the production of the mycobactins is susceptible to differences in the growth conditions. Alternatively, it is possible that some partial modification of the mycobactin molecule may have been affected by slight differences in our extraction and purification procedures.

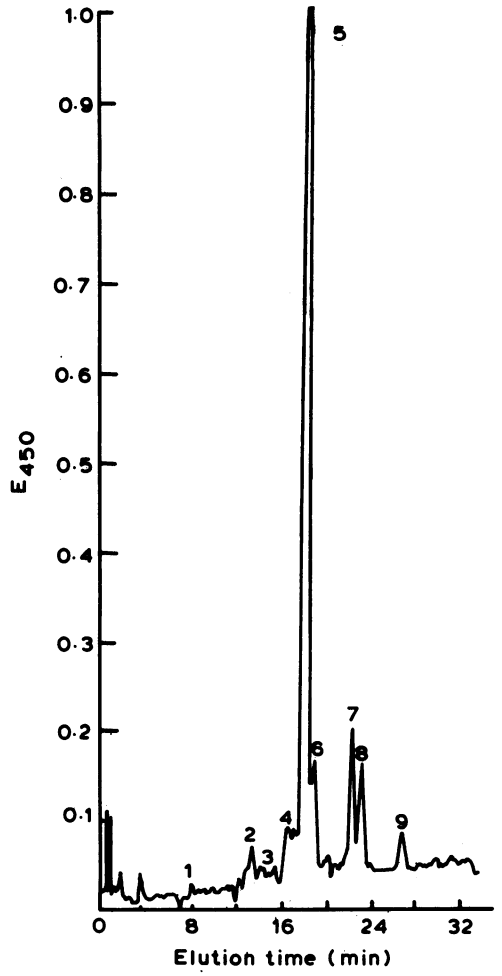


FIG. 5. Reverse phase HPLC of *M. paratuberculosis* NADC 18 mycobactins on a Lichrosorb RP-8 column with a gradient of 80% (vol/vol) methanol in water, rising to 100% methanol in 30 min. Operating conditions are described in the text. Numbers on peaks correspond to five identifiable fractions.

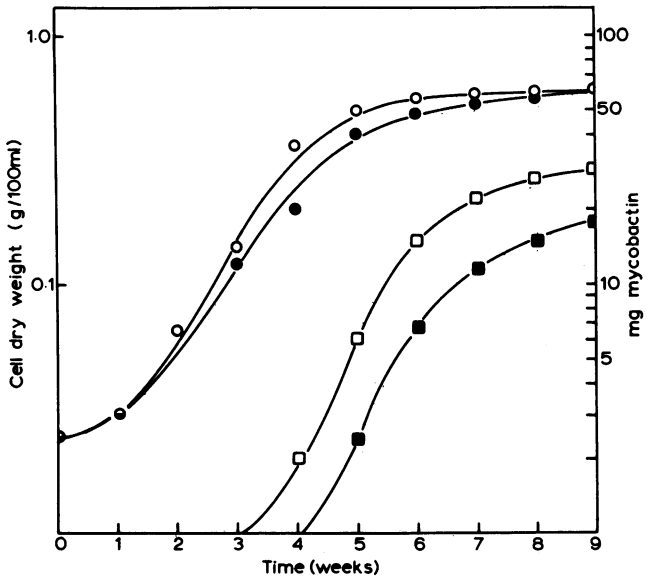


FIG. 6. Growth and mycobactin production by *M. paratuberculosis* NADC 18 in iron-deficient chemically defined media. Cell dry weight (○) and milligrams of mycobactin (□) in medium described by Merkal and McCullough (10). Cell dry weight (●) and milligrams of mycobactin (■) in medium described by Ratledge and Hall (15).

Effect of growth media and conditions on mycobactin formation. As far as was possible, we attempted to repeat the conditions of growth and of mycobactin extraction from strain NADC 18 as closely as possible to those described by Merkal and McCullough (10), although for completeness other conditions were also examined. *M. paratuberculosis* NADC 18 (10^6 cells) was used to inoculate liquid cultures (100 ml) of glycerol-asparagine-salts medium, Dubos broth, Reids medium, Middlebrook 7H9 and 7H11, Stuarts semisolid medium (which contained 0.75% agar), and the defined medium of Merkal and McCullough (10). The cultures were incubated without shaking at 38°C, and samples were taken weekly. At the same time the production of mycobactin on solid medium by the method of Hall and Ratledge (4) was also examined. The mycobactins were extracted from the samples and compared by TLC. No differences in the R_f values and relative proportions of the mycobactins were observed between culture media. However, the medium described by Merkal and McCullough (10) gave improved yields of mycobactin when compared with the usual medium (15) for mycobactin production (Fig. 6). Therefore, we conclude that, apart from differences in the overall rate of production, the types of mycobactin produced are not affected by changes in the growth conditions.

Mycobactin J and "J" (the major mycobactin component isolated from *M. paratuberculosis* NADC 18 grown in our laboratory) were held in 0.1, 1.0, or 10 M HCl or 0.1, 1.0, or 10 M NaOH at 37°C. Samples were removed at 0, 5, 15, 30, and 60 min and after 20 h. The pH was then returned to 7 to return the mycobactins to the ferri-form after acid treatment, and the mycobactin was examined by TLC. Hydrolysis of

the ester links of the mycobactins to give cobactin plus mycobactic acid (18) was observed, but no changes of the R_f values of the remaining mycobactin were seen, thus leading us to conclude that a simple chemical transition of J to "J" was probably not the explanation for the observed differences between the two mycobactins.

NMR spectroscopy. Examination of desferri-mycobactin "J" by 400-MHz NMR spectroscopy showed the protons common to the core structure of mycobactin. The remaining resonance peaks were designated as the R groups that are known to vary among mycobacteria (19) (Table 1). From these determinations we found no difference between the structure of mycobactin "J" and that of the major component of the *M. intracellulare* M12 mycobactin described above. There was no evidence that an isopropyl group, deduced from a low-resolution, 60-MHz spectrum of mycobactin J (9), was present at the R position of mycobactin "J." Therefore, we regarded mycobactin "J" and *M. intracellulare* mycobactin to be identical.

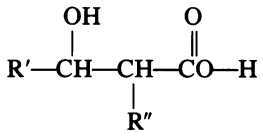
DISCUSSION

Each of the test organisms produced at least five mycobactins, as determined by TLC. Other mycobacteria, such as *M. fortuitum*, also produce multiple mycobactin components (19), but each component has only one long alkyl chain, which may be positioned at either R1 or R4. The presence of two alkyl chains in the major components of the MAIS mycobactins and in the *M. paratuberculosis* mycobactins isolated in our laboratory at R1 and R4 therefore is a novel feature of these mycobactins. The presence of this second alkyl chain could help to explain why MAIS mycobactins are more mobile in TLC solvent systems A and B than are the mycobactins from other mycobacteria (1, 16).

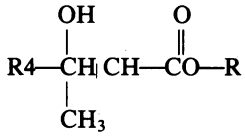
From the X-ray crystallographic studies (6) of ferri-mycobactins, we deduced that if the MAIS mycobactins have the same configuration of iron-binding centers as those previously described for mycobactin P, the two alkyl chains of these mycobactins will be approximately parallel to each other. The molecule therefore could form a stronger attachment to the mycobacterial cytoplasmic membranes than a mycobactin with only one alkyl chain and, perhaps, have a more specific orientation or occupy a more specific location within the cell envelope (17).

Separation of the MAIS mycobactin components by TLC gave two main products that were shown by NMR analysis to differ structurally in the core part of the molecule. Differences in the length of the R1 alkyl chain could not be determined by NMR analysis. However, when the components separated by TLC were examined by HPLC, each had an elution pattern that was typical of all of the MAIS mycobactins examined, although for the *M. scrofulaceum* mycobactins, the retention times were slightly longer. Thus the HPLC separation was deduced to be based on the differences in the properties of the R1 alkyl chains, whereas the separation by TLC with solvent system C was based on differences in the polar core of the mycobactin molecule. This was further reinforced by the observed delay in the HPLC retention times of the *M. scrofulaceum* mycobactins. These mycobactins differed from the other MAIS mycobactins by the position of the unsaturated bond in the R1 chains, as shown by NMR analysis.

The finding of a second alkyl chain at the R4 position of the major component of MAIS mycobactins raises the question of how this part of the molecule may be synthesized. However, a comparison of the structures of the cobactin with that of mycolic acid, the major fatty acid of the mycobacteria (12), shows that both can be regarded as β-hydroxy fatty acids. This would then suggest that cobactin may be synthesized by the same mechanisms used in the synthesis of mycolic acid, that is, by condensation of an alkanoic acid (propanoic acid for mycobactin, a C_{24} or C_{26} fatty acid for mycolic acid) with a further fatty acid (a C_{12} fatty acid for mycobactin and a C_{54}-C_{60} meromycolate for mycolic acid), as follows:



e.g., mycolic acid, in which R′ is a long alkyl chain (up to C_{60}) and R″ is a C_{22} or C_{24} chain.



e.g., cobactin, in which R4 is a long alkyl chain (~C_{10}), and R is the cyclohydroxylysine moiety (Fig. 1).

It was of considerable interest to examine the mycobactin from a strain of *M. paratuberculosis* which before this had required mycobactin for growth but had reverted, in common with strains of *M. avium*, from wood pigeons, to mycobactin production. The examination of *M. paratuberculosis* NADC 18 mycobactins by Merkal and co-workers (8, 10, 11) indicates that it has a unique structure clearly different from that produced by *M. avium*. Although the mycobactin as isolated and supplied to us by R. S. Merkal remains distinctive, the mycobactin as produced by our own cultivation of the designated species is the same as that of mycobactin Av. Other mycobactins, obtained from previously mycobactin-dependent *M. paratuberculosis* C57/2 and 465-BC(W), were also the same as mycobactin Av.

Mycobactin J, as isolated and described by Merkal and co-workers (8, 10, 11), was kindly supplied to us by R. S. Merkal. The major component ran as a single spot on TLC, corresponding to a minor (<10%) mycobactin component of the material isolated by us from *M. paratuberculosis* NADC 18. It is not unusual for mycobacteria to produce more than one mycobactin, and this can be seen easily by TLC. In some cases approximately equal amounts of different mycobactins may be produced; in others there is a major component and one or more minor ones. The proportions of the various mycobactins from a single organism do not appear to change with different cultural procedures (see above [6]).

We cannot explain how these differences between mycobactin J and "J" might have arisen, and we were unable to modify the chemical constitution of mycobactin "J" either by changes in growth conditions or by chemical means. Although we used various means of isolating mycobactin "J" and attempted to follow as closely as possible the published methods of Merkal and co-workers (8, 10, 11) for the growth of the organism and for the isolation of the mycobactin J, we are forced to conclude that there must be some crucial difference in our respective protocols.

These differences between the structures of mycobactin J and "J" would then lead to different conclusions. Because mycobactin J is a distinctive molecule, it could be argued the

M. paratuberculosis represents a distinctive chemotaxin. However, with our view being that mycobactin "J" is identical to mycobactin Av, we conclude that *M. paratuberculosis* should be included in the MAIS group of organisms and is no more than a fastidious subspecies of *M. avium*.

The exact mechanism by which mycobactin synthesis is switched on after several generations of growth on laboratory culture media has yet to be elucidated, but it may explain the differences in the results between the two laboratories. However, despite these differences, we are able to confirm that strain NADC 18 and other strains of *M. paratuberculosis* [i.e., C57/2 and 465 BC(W)] possess the ability to produce mycobactin.

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