Equivalence of Mycobactins from *Mycobacterium senegalense*, *Mycobacterium farcinogenes* and *Mycobacterium fortuitum*

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Mycobactins were isolated from five strains designated *Mycobacterium farcinogenes* and a similar number designated *Mycobacterium senegalense* following growth under conditions of iron-limitation. These lipid-soluble iron-chelating compounds were characterized by a combination of thin-layer and high-performance liquid chromatography. The mycobactins from both the slow-growing *M. farcinogenes* and the rapidly-growing *M. senegalense* strains proved impossible to differentiate both from each other and from those produced by strains of *Mycobacterium fortuitum*, indicating a close relationship between all three species. However, *Nocardia farcinica*, previously implicated with the bovine farcy strains, produced a different mycobactin which was easily distinguished by thin-layer chromatography alone.

INTRODUCTION

The classification and identification of those actinomycetes causing bovine farcy, a serious disease of Zebu cattle in Africa (Mostafa, 1966; Chamoiseau, 1969, 1972) has proved to be a complex taxonomic problem which still requires some clarification. Lipid analysis (Chamoiseau & Asselineau, 1970) showed that mycolic acids typical of the genus *Mycobacterium* were produced by these organisms and later serological studies by Ridell (1975) confirmed this view. This evidence was perhaps surprising since the causal agent of bovine farcy had long been considered to be *Nocardia farcinica*, at that time the type strain of the genus *Nocardia* (Nocard, 1888; Trevisan, 1889). Chamoiseau (1973) proposed the name *Mycobacterium farcinogenes* for the farcy strains and recognized two subspecies, *tchadense* and *senegalense*. However, differences in growth rate, DNA homology, chemical activity and pathogenicity later allowed these subspecies to be given full species status as *Mycobacterium farcinogenes* and *Mycobacterium senegalense* (Chamoiseau, 1979). Recent serological and chemical studies have questioned this division and also demonstrated a close relationship of the designated species with *Mycobacterium fortuitum* (Ridell, 1981, 1983; Ridell *et al.*, 1982; Ridell & Goodfellow, 1983; Minnikin & Goodfellow, 1980).

In this study we have compared the mycobactins produced by representative strains of *M. farcinogenes*, *M. senegalense*, *M. fortuitum* and *N. farcinica* in order to clarify the relationships between these taxa. Mycobactins, which are lipid-soluble iron-chelating compounds characteristic of the genera *Mycobacterium* and *Nocardia*, have recently been confirmed as excellent chemotaxonomic markers for *Mycobacterium* and related taxa based on their species-specificity and highly conserved nature (Hall & Ratledge, 1984).

METHODS

Strains. The sources of the test strains are given in Table 1.

Growth and maintenance. Strains were maintained on glucose/yeast extract/agar slopes (Gordon & Mihm, 1962) or as dense suspensions of cells frozen in 20% (v/v) glycerol at -20°C (Wellington & Williams, 1978). To encourage formation of mycobactin organisms were grown on either glycerol/asparagine/agar or glucose/yeast extract/agar as previously described (Hall & Ratledge, 1982).

Table 1. *Organisms grown under conditions of iron-limitation for mycobactin production*

Lab. no.*	Designation and source
M725	<i>M. farcinogenes</i> , M. Ridell, N73; M. Magnusson, Statens Seruminstitut, Copenhagen, Denmark, 749; bovine farcy, Farcha Laboratory, Fort Lamy, Chad.
N719	<i>M. farcinogenes</i> , M. Ridell, BF12; M. P. Lechevalier, Rutgers University, New Brunswick, NJ, USA, 1225; bovine farcy, Farcha Laboratory, Fort Lamy, Chad.
N710	<i>M. farcinogenes</i> , M. Ridell, BF2, University of Gothenburg, Sweden; R. E. Gordon, Rutgers University, New Brunswick, NJ, USA, 1226; bovine farcy, Farcha Laboratory, Fort Lamy, Chad.
M269	<i>M. farcinogenes</i> .
N711	<i>M. farcinogenes</i> .
N718	<i>M. senegalense</i> , M. Ridell, BF10; M. P. Lechevalier, 1364; bovine farcy, Dakar, Senegal.
N723	<i>M. senegalense</i> .
N728	<i>M. senegalense</i> .
N721	<i>M. senegalense</i> .
M263	† <i>M. senegalense</i> NCTC 10956.
M368	<i>M. fortuitum</i> .
M369	<i>M. fortuitum</i> , M. Ridell, X42, ATCC 6841.
-	† <i>N. farcinica</i> NCTC 11134 (ATCC 3318).

* Laboratory numbers correspond to the collection of Dr M. Goodfellow, Department of Microbiology, University of Newcastle upon Tyne, UK. Full details of strain histories not given here appear in Ridell & Goodfellow (1983). NCTC, National Collection of Type Cultures, London, UK.

† Type strain.

Isolation and characterization of mycobactins. Bacteria were harvested, mycobactins isolated and their yields determined after partial purification as previously described (Hall & Ratledge, 1982, 1984). Mycobactins were analysed by thin-layer chromatography (TLC), in a single dimension, using various adsorbents and solvents: system I, silica gel G, 20 cm × 20 cm (Analtech, Newark, USA) developed with petroleum spirit (b.p. 60–80 °C)/ethyl acetate/*n*-butanol (2:3:3, by vol.); system II (Merck, product no. 11844), silica gel 60, 10 cm × 20 cm with a 2.5 cm × 10 cm concentrating zone, developed with petroleum spirit/ethyl acetate/*n*-butanol (2:3:3, by vol.); system III (Merck, product no. 13748), high performance thin-layer plates, 10 cm × 10 cm, including a 2.5 cm × 10 cm concentrating zone (BDH), developed with petroleum spirit/ethyl acetate/*n*-butanol (2:3:3, by vol.); system IV, alumina GF, 20 cm × 20 cm (Analtech), developed with cyclohexane/*n*-butanol (9:1, v/v); system V, plates as for system I but using methanol/ethyl acetate (4:1, v/v) for development; system VI, plates as for system I but using propan-1-ol as solvent (Hall & Ratledge, 1984).

HPLC analyses of the mycobactins were done with a Spectra-Physics (Santa Clara, Calif., USA) SP 8000 instrument. A solvent gradient, changing over 30 min from methanol/water (80:20, v/v) to methanol alone, was used with a flow rate of 2 ml min⁻¹ and a column temperature of 50 °C. A Lichrosorb RP18 reverse-phase column (4 mm × 250 mm) (Jones Chromatography, Llanbradach, Mid-Glamorgan, UK) was used as previously described (Hall & Ratledge, 1984) with the eluate monitored at 450 nm using a UV-visible detector (Spectra-Physics model 770). All solvents were of HPLC grade quality. The relative percentages of the various peaks were calculated by the electronic integrator of the instrument.

RESULTS AND DISCUSSION

Mycobactins were isolated from all of the test strains (Table 1) following their growth under conditions of iron-limitation on solid medium (Hall & Ratledge, 1982). These compounds were then characterized by both TLC and HPLC, an approach which has been shown to be a powerful technique for resolving mycobactins from various mycobacteria (Hall & Ratledge, 1984).

Analysis by single-dimensional TLC (Table 2) showed that mycobactins isolated from *M. farcinogenes*, *M. senegalense* and *M. fortuitum* could not be differentiated by any of the six TLC systems used. As shown previously (Hall & Ratledge, 1984), multiple strains of the same species produced mycobactins with identical TLC migration values, again highlighting the intra-species consistency of these molecules. The mycobactin isolated from *N. farcinica* was however distinct and could be readily differentiated from those of the other species examined by any of the TLC systems except system IV. This distinction of *N. farcinica* from *M. farcinogenes* and *M. senegalense* confirms earlier work which used lipid and immunological analyses (Ridell *et al.*, 1982). In addition, the mycobactin produced by *N. farcinica* also allows its differentiation from other *Nocardia* species (R. M. Hall & C. Ratledge, unpublished results).

Table 2. *Thin-layer chromatography of mycobactins isolated from test strains*

The R_F values obtained from TLC with different solvent systems are given.

Test strain	R_F values with TLC system*:					
	I	II	III	IV	V	VI
<i>M. farcinogenes</i> M269†	0.46	$\frac{0.34}{0.31}$	$\frac{0.41}{0.40}$	0.34	0.87	0.51
<i>N. farcinica</i> ATCC 3318	0.31	0.20	0.18	St	0.67	0.33

* $\frac{x}{y}$ refers to a double spot with both R_F values given; St, streak.

† Mycobactins from all other strains of *M. farcinogenes*, *M. senegalense* and *M. fortuitum* co-chromatographed with that from *M. farcinogenes* M269.

Table 3. *Yields of mycobactins and their analysis by HPLC*

A Lichrosorb RP18 reverse-phase column was used with a solvent gradient changing from methanol/water (80:20, v/v) over 30 min to methanol alone. Only peak areas >2% of the final chromatogram are shown.

Test strain	Mycobactin yield (%)*	Retention time (s) of mycobactins (peak area, percentage of total mycobactins)					
<i>M. farcinogenes</i> M269	2.2	480 (2.0)	530 (2.1)	1025 (15.5)	1074 (8.6)	1285 (41.3)	1395 (30.0)
<i>M. farcinogenes</i> M710	1.1	478 (2.5)	527 (2.0)	1020 (15.6)	1064 (8.5)	1280 (38.9)	1389 (31.2)
<i>M. farcinogenes</i> M711	1.2	479 (2.9)	525 (2.3)	1021 (14.5)	1065 (9.3)	1284 (40.1)	1390 (30.1)
<i>M. farcinogenes</i> N719	1.0	482 (2.0)	532 (2.0)	1029 (18.5)	1082 (9.1)	1290 (41.2)	1397 (27.1)
<i>M. farcinogenes</i> M725	0.9	485 (2.4)	534 (2.1)	1031 (19.5)	1085 (9.0)	1292 (38.4)	1399 (27.5)
<i>M. senegalense</i> M263	3.6	486 (3.4)	536 (2.4)	1028 (16.4)	1082 (8.0)	1289 (42.0)	1401 (27.5)
<i>M. senegalense</i> N718	3.8	490 (4.0)	538 (2.5)	1029 (18.3)	1083 (8.9)	1294 (41.6)	1400 (25.4)
<i>M. senegalense</i> N723	3.5	491 (3.2)	539 (2.2)	1022 (16.5)	1078 (7.4)	1293 (41.4)	1392 (25.0)
<i>M. senegalense</i> N728	3.9	485 (3.1)	533 (2.0)	1028 (15.5)	1080 (6.9)	1284 (40.5)	1398 (30.4)
<i>M. senegalense</i> N721	3.8	489 (3.6)	537 (2.2)	1028 (15.1)	1076 (7.0)	1288 (40.7)	1399 (31.2)
<i>M. fortuitum</i> M368	3.4	480 (7.1)	531 (3.4)	1025 (11.9)	1076 (5.2)	1290 (37.4)	1389 (32.4)
<i>M. fortuitum</i> M369	3.9	479 (6.9)	530 (2.8)	1022 (12.0)	1070 (5.7)	1279 (35.4)	1380 (30.8)
<i>N. farcinica</i> ATCC 3318	2.8	373 (44.0)	470 (6.2)	589 (41.0)	850 (8.3)		

* Expressed as percentage of cell dry weight.

Isolated mycobactins were then examined by reverse-phase HPLC, which resolves an individual mycobactin into a number of components according to the length of the major alkyl chain substituent (Ratledge & Ewing, 1978). A 'finger print' for each mycobactin is provided which allows accurate identification of the producing organism (Hall & Ratledge, 1984). As before, the mycobactins from *M. farcinogenes*, *M. senegalense* and *M. fortuitum*, which could not be differentiated by TLC, were indistinguishable from each other (Fig. 1, Table 3). Previously,

Fig. 1. HPLC of mycobactins isolated from (a) *M. senegalense* M263, (b) *M. farcinogenes* M269, (c) a mixture of mycobactins from *M. senegalense* M263, *M. farcinogenes* M269 and *M. fortuitum* NCTC 10394 and (d) *N. farcinia* ATCC 3318. The mycobactins were separated using a reverse-phase column (250 mm $\times$ 4 mm Lichrosorb RP18) with a solvent gradient changing from methanol/water (80:20, v/v) to methanol alone over 30 min. A flow rate of 2 ml min⁻¹ and a column temperature of 50 °C were maintained throughout. Peaks are labelled with retention times in seconds; quantitative data are given in Table 3.

M. fortuitum strains has been shown to give a three doublet 'finger print' with reverse-phase HPLC (Hall & Ratledge, 1984) and this was confirmed. Although there were some minor differences in the relative proportions of some of the peaks (Table 3), these were negligible compared to the overall similarity of the mycobactins. When mycobactin samples isolated from these three species were mixed and re-examined by HPLC, the same three doublet pattern was still seen, thereby confirming that the mycobactins were identical (Fig. 1c). Thus on the basis of their mycobactins the three species *M. farcinogenes*, *M. senegalense* and *M. fortuitum* are judged to be equivalent. HPLC examination of the mycobactin synthesized by *N. farcinia* again confirmed its dissimilarity from the remaining test strains (Fig. 1d). The yields of the

mycobactins (Table 3) from representatives of *M. farcinogenes* are generally lower than those from strains of *M. senegalense* and *M. fortuitum*, perhaps reflecting the higher growth rate of the latter two species.

The results of this work confirm and extend the conclusions from previous work using lipid and serological analysis. On the basis of lipid analysis (Ridell *et al.*, 1982), *M. farcinogenes* and *M. senegalense* could not be differentiated; each contained α -mycolates and characteristic polar mycolates, derived from epoxymycolic acids, a pattern also found in *M. smegmatis*, *M. fortuitum* and '*Mycobacterium peregrinum*' (Minnikin *et al.*, 1984). Mycolic acid TLC patterns may not be sensitive enough to permit species definition however (Minnikin & Goodfellow, 1980). Serological analysis also indicates that *M. farcinogenes* and *M. senegalense* are closely related to each other and to *M. fortuitum* and to a lesser extent to *M. phlei* and *M. smegmatis* (Ridell *et al.*, 1979, 1982).

The mycobactins synthesized by *M. fortuitum*, *M. phlei* and *M. smegmatis* have previously been shown to be clearly different and characteristic of three distinct taxa (Hall & Ratledge, 1984). In contrast, the mycobactins isolated from *M. fortuitum*, *M. farcinogenes* and *M. senegalense* are indistinguishable using the present TLC and HPLC systems. Recent numerical studies (Ridell & Goodfellow, 1983) have shown that *M. senegalense* has a high overall similarity with *M. fortuitum* while the slower-growing *M. farcinogenes* cluster appeared to be distinct and deserving full species status. Preliminary DNA homology data (Baess, 1982) support the recognition of three distinct taxa corresponding to *M. farcinogenes*, *M. fortuitum* and *M. senegalense*. In view of the previously established strong correlation between the mycobactin-based chemotaxa and species level within the genus (Hall & Ratledge, 1984; Snow, 1970), the present evidence provides no support for the separation of *M. farcinogenes*, *M. senegalense* and *M. fortuitum* into distinct species. Further detailed comparisons of the interrelationships between these three taxa should be made to determine the true significance of the present results.

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