Classification of *Mycobacterium farcinogenes* and *Mycobacterium senegalense* by Immunodiffusion and Thin-layer Chromatography of Long-chain Components

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Comparative immunodiffusion studies and thin-layer chromatographic analyses of whole-organism acid methanolysates were performed on 37 strains of *Mycobacterium farcinogenes*, *Mycobacterium senegalense* and *Nocardia farcinica*. The latter were clearly distinguished from the mycobacteria in containing a single mycolic acid methyl ester and showing more precipitinogens with nocardial than with mycobacterial and rhodococcal reference systems. The distribution of precipitinogens showed that *M. farcinogenes* and *M. senegalense* were very closely related and that both showed a greater affinity to *Mycobacterium fortuitum* than to any of the other established species of *Mycobacterium* tested. The complex pattern of α -mycolates and characteristic polar mycolates found in both *M. farcinogenes* and *M. senegalense* has only previously been found in *M. fortuitum* and *Mycobacterium smegmatis*.

INTRODUCTION

The application of modern taxonomic methods has clarified relationships within and between the genera *Mycobacterium* and *Nocardia*, and has led to improved circumscription of these taxa (Bradley, 1973, 1975; Lechevalier, 1976; Goodfellow & Minnikin, 1977, 1981, 1982; Goodfellow & Wayne, 1982; Mordarski *et al.*, 1977, 1978; Minnikin & Goodfellow, 1980; Lind & Ridell, 1982). A number of taxonomic problems do, however, remain and these include the status and relationships of the taxa *Nocardia farcinica*, *Mycobacterium farcinogenes* and *Mycobacterium senegalense*.

Strains originally labelled *N. farcinica* fall into at least two distinct groups, one of which encompasses the causal agent of bovine farcy. Thus, strains such as *N. farcinica* ATCC 3318 are closely related to *Nocardia asteroides* (Gordon & Mihm, 1959, 1962*a*; Ridell, 1975; Schaal & Reutersberg, 1978; Williams *et al.*, 1980), whereas others, such as NCTC 4524, a strain supposedly identical to ATCC 3318, have many properties in common with mycobacteria and are now classified accordingly (Anderson & Bradley, 1961; Chamoiseau & Asselineau, 1970; Lanéele *et al.*, 1971; Lechevalier *et al.*, 1971; Ridell & Norlin, 1973; Ridell, 1975; Magnusson, 1976; Collins *et al.*, 1977; Orchard & Goodfellow, 1980). Gordon & Mihm (1962*a*) were unable to separate *N. farcinica* ATCC 3318 from typical strains of *N. asteroides* but, in contrast, Magnusson & Mariat (1968) found this and related strains to be clearly distinguishable from *N. asteroides*. In a subsequent study, Tsukamura (1969) recovered strain ATCC 3318 in a homogeneous phenon that was designated *N. farcinica* Trevisan.

Chamoiseau (1973) introduced the taxon *Mycobacterium farcinogenes*, and recognized two subspecies, *tchadense* and *senegalense*, each of which accommodated strains able to cause

Table 1. *Designation and sources of test strains*

M260	<i>Mycobacterium farcinogenes</i> ; M. P. Lechevalier, Rutgers University, New Brunswick, U.S.A.; Institut Pasteur (IP) 736; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N166
M261	<i>M. farcinogenes</i> ; M. P. Lechevalier; IP 740; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N167
*M262	<i>M. farcinogenes</i> ; M. P. Lechevalier; M. Goodfellow; NCTC 10955; M. Ridell, N168
M269	<i>M. farcinogenes</i> ; E. H. Runyon, Tuberculosis Research Institute, Pretoria, South Africa, R422; IP 735; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N204
M271	<i>M. farcinogenes</i> ; E. H. Runyon, R415; R. E. Gordon, 1226; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N193
M272	<i>M. farcinogenes</i> ; E. H. Runyon, R425; IP 738; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N194
M273	<i>M. farcinogenes</i> ; E. H. Runyon, R414; IP 739; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N212
M275	<i>M. farcinogenes</i> ; E. H. Runyon, R427; IP 742; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N213
M276	<i>M. farcinogenes</i> ; E. H. Runyon, R428; IP 743; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N195
M277	<i>M. farcinogenes</i> ; E. H. Runyon, R429; IP 744; bovine farcy, Farcha Laboratory, Fort Lamy, Chad; M. Ridell, N196
M280	<i>M. farcinogenes</i> ; E. H. Runyon, R147; P. Perreau, MA 75; M. Ridell, N214
N709	<i>M. farcinogenes</i> ; M. Ridell, BF1; R. E. Gordon, 1223; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
N711	<i>M. farcinogenes</i> ; M. Ridell, BF3; R. E. Gordon, 1242; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
N715	<i>M. farcinogenes</i> ; M. Ridell, BF7; M. P. Lechevalier, 1224; bovine farcy, Farcha Laboratory, Fort Lamy, Chad
N716	<i>M. farcinogenes</i> ; M. Ridell, BF8; M. P. Lechevalier, 1243; bovine farcy, Sudan
N720	<i>M. farcinogenes</i> ; M. Ridell, BF13; M. P. Lechevalier, 1244; bovine farcy, Sudan
M259	<i>Mycobacterium senegalense</i> ; M. P. Lechevalier; M. Goodfellow, W34; NCTC 4524; M. Ridell, N165
*M263	<i>M. senegalense</i> ; M. P. Lechevalier; M. Goodfellow; NCTC 10956; M. Ridell, N169
M265	<i>M. senegalense</i> ; E. H. Runyon, R408; R. E. Gordon, 1361; IP 397; bovine farcy, Dakar, Senegal; M. Ridell, N184
M279	<i>M. senegalense</i> ; E. H. Runyon, R148; P. Perreau, MA378; M. Ridell, N180
M282	<i>M. senegalense</i> ; E. H. Runyon, R455; P. Perreau, D1; M. Ridell, N176
M283	<i>M. senegalense</i> ; E. H. Runyon, R456; P. Perreau, D2; M. Ridell, N177
N696	<i>M. senegalense</i> ; M. Ridell, N33; I. Juhlin, 396/193
N712	<i>M. senegalense</i> ; M. Ridell, BF4; R. E. Gordon, 1360, 5702; IP 396; bovine farcy, French West Africa
N714	<i>M. senegalense</i> ; M. Ridell, BF6; R. E. Gordon, 1363, 434C; bovine farcy, Dakar, Senegal
N721	<i>M. senegalense</i> ; M. Ridell, BF14; M. P. Lechevalier, 931; bovine farcy, Dakar, Senegal
N723	<i>M. senegalense</i> ; M. Ridell, N56; R. N. Bönicke, SN5701
N727	<i>M. senegalense</i> ; M. Ridell, N122; M. Magnusson, 875; bovine farcy, Dakar, Senegal
N728	<i>M. senegalense</i> ; M. Ridell, N123; M. Magnusson, 876; isolated from same animal as N727
N729	<i>M. senegalense</i> ; E. H. Runyon, 410; R. E. Gordon, 1364; M. Ridell, N124
*M258	<i>Nocardia farcinica</i> ; M. P. Lechevalier; ATCC 3318; M. Ridell, N164
*N699	<i>N. farcinica</i> ; M. Ridell, N58; R. N. Bönicke, EB1957; ATCC 3318
*N700	<i>N. farcinica</i> ; M. Ridell, N66; M. Magnusson, 752; R. E. Gordon; ATCC 3318 (<i>N. asteroides</i>)
N701	<i>N. farcinica</i> ; M. Ridell, N67; M. Magnusson, 753; ATCC 3399 (<i>N. asteroides</i>)
N704	<i>N. farcinica</i> ; M. Ridell, N118; M. Magnusson, 654; ATCC 6864, NCTC 1935; isolated from a rabbit, East Sumatra, 1925
N705	<i>N. farcinica</i> ; M. Ridell, N119; M. Magnusson, 844; isolated from a case of nocardiosis in Sweden
N707	<i>N. farcinica</i> ; M. Ridell, N125; M. Magnusson, 878

* Type strain.

bovine farcy (Chamoiseau, 1969, 1972). The subspecies were subsequently raised to species status as *Mycobacterium farcinogenes* and *Mycobacterium senegalense*, respectively (Chamoiseau, 1979). Strains of the latter when compared with the former are rapid-growers as opposed to slow-growers, are more active biochemically, contain a characteristic mycoside C, cause a more generalized peritonitis in guinea pigs, but do show appreciable DNA homology with *M. farcinogenes*. Little is known of the relationships of *M. farcinogenes* and *M. senegalense* to established *Mycobacterium* species, though the agents of bovine farcy can be

distinguished by their characteristic pathogenicity for guinea pigs, ability to form a stable mycelium and positive malonamidase reaction (Chamoiseau, 1979). Preliminary immunodiffusion (Ridell *et al.*, 1979) studies indicate an affinity between the bovine farcy organisms and *Mycobacterium fortuitum* and *Mycobacterium smegmatis*.

In the present study, representatives of *M. farcinogenes*, *M. senegalense* and *N. farcinica* were the subject of comparative immunodiffusion and lipid analyses designed to determine the relationships of these taxa to one another and to related actinomycetes.

METHODS

Strains. The sources of the 37 strains included in both the immunodiffusion and lipid analyses are given in Table 1. In addition, ten representative strains of the genus *Mycobacterium*, two of *Nocardia* and one of *Rhodococcus* (see Tables 2 and 3) were included in the serological analyses for comparative purposes.

Analysis of long-chain components. The test strains were maintained on glucose yeast extract agar at room temperature (Gordon & Mihm, 1962*b*). Biomass for the lipid analyses was prepared by growing organisms in shake flasks of modified Sauton's medium (Mordarska *et al.*, 1972) for 7–14 d at 30 °C. The strains were checked for purity at maximum growth, killed with formaldehyde (1%, v/v), harvested by centrifugation, washed with distilled water and freeze-dried.

Freeze-dried bacteria (50 mg) were degraded by treatment at 75 °C with a mixture (3 ml) of methanol/toluene/sulphuric acid (30:15:1, by vol.) for 16 h (Minnikin *et al.*, 1980). Long-chain components were extracted with two portions of petroleum ether (b.p. 60–80 °C) and traces of acid were removed by passage through a short column of ammonium hydrogen carbonate. Thin-layer chromatographic (t.l.c.) analyses of the extracts were performed on 10 × 10 cm pieces of Merck 5554 silica gel 60 F₂₅₄ aluminium sheets or on 20 × 30 cm layers (0.5 mm) of Merck 7739 silica gel 60 HF₂₅₄. Chromatography in a single dimension involved a single development with petroleum ether/acetone (95:5, by vol.); for two-dimensional analysis, a triple development with the same mixture in the first direction was followed by a single development with toluene/acetone (97:3, by vol.) (Minnikin *et al.*, 1980) in the second direction. Separated components were revealed on aluminium-backed sheets by heating at 120 °C for 15 min after spraying with 10% ethanolic molybdophosphoric acid, while for layers spread on glass, spraying with 50% aqueous sulphuric acid was followed by charring at 180 °C for 20 min.

Comparative immunodiffusion analyses. Antigens were prepared from extracts of disintegrated cell mass as described previously (Ridell & Norlin, 1973). Thirteen reference precipitation systems (Table 2) were employed including those prepared from the type strains of *M. farcinogenes* (M262, NCTC 10955) and *M. senegalense* (M263, NCTC 10956), and from *M. senegalense* M696. The other ten reference systems and the methods employed for their preparation have been described previously (Ridell & Norlin, 1973; Ridell, 1975; Ridell *et al.*, 1979). The serological analyses were performed using a microplate modification (Wadsworth, 1962) of the immunodiffusion technique of Ouchterlony (1958, 1962). For each combination of test antigen and reference antiserum, two results were recorded: the first figure represents the total number of precipitation lines obtained in the reaction between the test antigen and the reference antiserum; the second figure represents the number of precipitinogens of the test antigen that could be identified by means of the reference system (Ridell, 1975; Ridell *et al.*, 1979).

RESULTS

Analysis of long-chain components

Single-dimensional t.l.c. of whole-organism acid methanolysates of the test strains (Table 1) indicated that the patterns of long-chain compounds from the *N. farcinica* strains were clearly distinct from those of *M. farcinogenes* and *M. senegalense*. Two-dimensional t.l.c. gave more precise separation of all of the components: representative examples, for *N. farcinica* M258 (ATCC 3318), *M. farcinogenes* M262 (NCTC 10955) and *M. senegalense* M263 (NCTC 10956) and N723, are given in Fig. 1. All of the *N. farcinica* strains (Table 1) gave simple patterns similar to that shown for strain M258 (Fig. 1). Such a pattern, consisting of non-hydroxylated fatty acid methyl esters, single mycolic acid methyl ester components, and occasionally characteristic long-chain alcohols (nocardols), is characteristic of *Nocardia sensu stricto* (Minnikin & Goodfellow, 1976, 1980; Minnikin *et al.*, 1980). The proportion of nocardols was highest in strain M258 but significant amounts were detected in the methanolysates from other strains.

Fig. 1. Two-dimensional t.l.c. of whole-organism acid methanolysates of *N. farcinica* M258 (ATCC 3318), *M. farcinogenes* M262 (NCTC 10955), *M. senegalense* M263 (NCTC 10956) and *M. senegalense* N723. A triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction was followed by a single development with toluene/acetone (97:3, v/v) in the second direction. Abbreviations: F, non-hydroxylated fatty acid methyl esters; X, possible long-chain alcohol ('nocardol'); A, α -mycolate; A', possible α' -mycolate; I, J, K, L, N, O, unknown long-chain components; ?, unidentified minor components.

All the strains (Table 1) of both *M. farcinogenes* and *M. senegalense* had general patterns of long-chain compounds similar to those recorded for the type strains M262 (NCTC 10955) and M263 (NCTC 10956) (Fig. 1), and copies of these have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, as Supplementary Publication No. SUP 28013 (5 pages). (Copies may be obtained from the BLLD on demand; wherever possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.)

The principal long-chain components present in *M. farcinogenes* and *M. senegalense* (Fig. 1) co-chromatographed with non-hydroxylated fatty acid methyl esters and mycolic esters similar to those found in methanolysates of *M. fortuitum* and *M. smegmatis* (Minnikin *et al.*, 1980). The least polar mycolates (A, A'; Fig. 1) are considered to be the so-called α - and

Table 2. Average number of precipitinogens revealed when the *M. farcinogenes*, *M. senegalense* and *N. farcinica* strains were examined by immunodiffusion analysis using mycobacterial, nocardial and rhodococcal reference systems

For each test combination, the first figure represents the average (to the nearest whole number) of the total number of precipitinogens revealed, and the second figure, in parentheses, represents the number of identified precipitinogens.

Test species	No. of precipitinogens in the reference system	No. of strains	Reference system													
			BCG, Swedish substrain	<i>M. microti</i> SG851	<i>M. kansasii</i> MS32	<i>M. scrofulaceum</i> MS18	<i>M. avium</i> MA4	<i>M. fortuitum</i> 456	<i>M. phlei</i> ATCC 19249	<i>M. farcinogenes</i> M262 (NCTC 10955)	<i>M. senegalense</i> M263 (NCTC 10956)	<i>M. senegalense</i> N696	<i>N. asteroides</i> ATCC 19247	<i>N. otitidis-caviae</i> ATCC 14629	<i>R. rubropertinctus</i> N32	
<i>M. farcinogenes</i>	16	...	10	8	10	10	7	10	8	8	8	8	7	10	7	
			4(1)	3(0)	3(0)	3(1)	4(1)	4(2)	4(1)	7(6)	7(5)	7(5)	2(0)	1(0)	2(0)	
<i>M. senegalense</i>	14		4(1)	3(0)	3(0)	3(1)	4(2)	5(3)	3(1)	5(5)	7(6)	6(5)	2(0)	2(0)	1(0)	
<i>N. farcinica</i>	7		1(0)	2(0)	1(0)	2(1)	2(1)	1(0)	1(0)	2(1)	3(0)	1(0)	4(3)	4(2)	2(0)	

Table 3. *Number of precipitinogens revealed when ten representative mycobacteria were examined by immunodiffusion analysis using one M. farcinogenes and two M. senegalense reference systems*

For each test combination, the first figure represents the total number of precipitinogens revealed, and the second figure, in parentheses, represents the number of identified precipitinogens.

Test strain	Reference system		
	<i>M. farcinogenes</i> M262 (NCTC 10955)	<i>M. senegalense</i> M263 (NCTC 10956)	<i>M. senegalense</i> N696
No. of precipitinogens in the reference system	...	8	8
BCG, Swedish substrain	3(1)	3(1)	2(1)
<i>M. microti</i> SG851	2(0)	1(1)	0(0)
<i>M. kansasii</i> ATCC 25221	3(1)	3(1)	1(1)
<i>M. scrofulaceum</i> ATCC 25214	3(1)	3(2)	0(0)
<i>M. avium</i> ATCC 19421	4(2)	3(0)	2(1)
<i>M. fortuitum</i> ATCC 6841	6(4)	6(4)	6(5)
<i>M. parafortuitum</i> ATCC 19686	4(1)	3(1)	4(1)
<i>M. phlei</i> ATCC 19249	4(2)	6(3)	4(0)
<i>M. smegmatis</i> ATCC 14468	6(3)	4(3)	6(3)
<i>M. vaccae</i> ATCC 15483	4(1)	3(1)	4(1)

α' -mycolates (Etémadi, 1967) which lack oxygen functions apart from the 3-hydroxy ester unit. The other most characteristic long-chain components found in all the methanolysates of *M. farcinogenes* and *M. senegalense* are the pairs of polar mycolates (I, J; N, O; Fig. 1) previously found only in *M. fortuitum* and *M. smegmatis* (Minnikin & Goodfellow, 1980; Minnikin *et al.*, 1980). In certain strains other minor long-chain components were observed, the most prominent being those (K, L) found in *M. senegalense* N723 (Fig. 1) which correspond in chromatographic behaviour to components detected previously in acid methanolysates of *M. fortuitum* and *M. smegmatis* (Minnikin *et al.*, 1980). Component K also appeared to be present in the methanolysates of *M. senegalense* M283, N696 and N712 co-occurring with smaller amounts of component L. The latter was also found in fairly significant amounts in methanolysates of *M. farcinogenes* M271, M272, M277, N709, N711, N715, N716 and N720 and to a lesser degree in *M. farcinogenes* M269, M273 and M280 and in *M. senegalense* N721, N727, N728 and N729.

Comparative immunodiffusion analyses

The 37 test strains were analysed by the comparative immunodiffusion technique employing ten mycobacterial, two nocardial and a single rhodococcal reference system (Table 2). The average numbers of precipitinogens shared by the test and reference strains are shown in Table 2. The *M. farcinogenes* and *M. senegalense* strains shared a higher number of precipitinogens with the mycobacterial reference strains than with the nocardial and rhodococcal ones; in contrast, the *N. farcinica* strains shared the most precipitinogens with the nocardial reference strains. The largest numbers of identified precipitinogens, either five or six, were observed when the *M. farcinogenes* and *M. senegalense* strains were analysed with the three corresponding reference systems, the next highest numbers being observed with the *M. fortuitum* reference system.

The numbers of precipitinogens found when representative mycobacteria were analysed with the *M. farcinogenes* and two *M. senegalense* reference systems are shown in Table 3. The highest numbers of revealed and identified precipitinogens were found in reactions between the type strain of *M. fortuitum* and the three reference systems, though several precipitinogens were also recorded between the latter and *M. smegmatis* ATCC 14468. It is also interesting

that, in the reaction between *Mycobacterium phlei* ATCC 19249 and the *M. farcinogenes* M262 (NCTC 10955) and *M. senegalense* M263 (NCTC 10956) systems, two and three precipitinogens were identified, respectively.

DISCUSSION

The *N. farcinica* strains, including the type strain ATCC 3318, were clearly distinguished from both *M. farcinogenes* and *M. senegalense* in the lipid and immunodiffusion analyses. Thus, these strains contained a single mycolic acid methyl ester and non-hydroxylated fatty acid methyl esters characteristic of true nocardiae (Minnikin & Goodfellow, 1980), and showed more precipitinogens on immunodiffusion with the nocardial reference systems than with the mycobacterial and rhodococcal reference systems (Table 2). These data add weight to the view that *N. farcinica* Trevisan is a good *Nocardia* species (Tsukamura, 1969; Ridell, 1975; Bradley & Mordarski, 1976; Orchard & Goodfellow, 1980).

Mycobacterial mycolates yield a number of characteristic patterns on t.l.c. analysis of whole-organism acid methanolysates (Minnikin & Goodfellow, 1980). The mycolates of *M. tuberculosis*, consisting of α -mycolates, ketomycolates and methoxymycolates, are an example of the first pattern; the second pattern, exemplified by *M. avium*, consists of α -mycolates, ketomycolates, ω -carboxymycolates and 2-eicosanol; in the third pattern, α - and α' -mycolates co-occur with characteristic unknown polar mycolates, as found for acid methanolysates of *M. fortuitum* and *M. smegmatis* (Minnikin *et al.*, 1980); finally, *M. chelonae* strains give a very simple mycolate pattern of two spots which correspond to a diunsaturated α -mycolate and lower molecular weight α' -mycolate (Minnikin *et al.*, 1982). The principal long-chain components of the *M. farcinogenes* and *M. senegalense* strains co-chromatographed with methyl mycolates and non-hydroxylated fatty acid methyl esters similar to those found in *M. fortuitum* and *M. smegmatis*. On the basis of the lipid data, therefore, *M. farcinogenes* and *M. senegalense* cannot be distinguished as each contains α -mycolates and characteristic polar mycolates previously only found in *M. fortuitum* and *M. smegmatis*.

The large number of identified precipitinogens demonstrated between the *M. farcinogenes* and *M. senegalense* strains in immunodiffusion analyses shows not only that they are closely related but is consistent with their classification in a single species (Ridell, 1974, 1981). The serological data also show that both the slow-growing *M. farcinogenes* and the fast-growing *M. senegalense* are closely related to *M. fortuitum*, and to a lesser extent to *M. phlei* and *M. smegmatis*, but can readily be distinguished from other established species of *Mycobacterium* (Tables 2 and 3). These findings are in good agreement with preliminary immunological data (Ridell *et al.*, 1979).

Both lipid and serological data indicate that *M. farcinogenes* and *M. senegalense* are closely related to one another and to *M. fortuitum*. The results of the serological analyses suggest that *M. farcinogenes* and *M. senegalense* might be classified as a single species, whereas t.l.c. analysis of mycobacterial mycolates does not always yield data sufficiently sensitive for species differentiation. Detailed studies using appropriate modern taxonomic techniques are required to determine whether or not *M. farcinogenes* and *M. senegalense* should remain as separate species.

Recently, Shigidi *et al.* (1980) isolated 21 strains from cattle with bovine farcy in the Sudan and assigned them to the genus *Nocardia* on the basis of chemical, physiological and serological criteria. The results of this interesting study need to be confirmed as the taxonomic data do not demonstrate unequivocally that the isolates are nocardiae. Thus, nocardiae and mycobacteria share many physiological properties. They have also several antigens in common and the presence or absence of a single unidentified precipitinogen cannot be used to distinguish between the genera *Mycobacterium* and *Nocardia*.

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