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NOCARDIA-LIKE MYCOBACTERIA ISOLATED FROM NATURAL HABITATS IN ZAIRE

by

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Summary — Forty slow-growing actinomycetes were isolated from a variety of natural habitats in Zaire. The organisms were acid-alcohol fast and formed a primary mycelium covered by abundant aerial hyphae. The majority of the isolates contained mycolic acids characteristic of mycobacteria and formed two homogenous groups on the basis of morphological, biochemical and physiological properties. These novel mycobacteria were clearly distinguishable from other non-pigmented slow growing mycobacteria.

KEYWORDS : Mycobacteria; Natural Habitats; Nocardia-like Mycobacteria; Mycobacterial Mycolic Acids; Zaire.

1. Introduction

Modern taxonomic methods have led to significant improvements in the classification of the genera *Mycobacterium* and *Nocardia* but have been less successful in yielding good characters for the separation of these and related nocardioform taxa (Goodfellow & Minnikin, 1977, 1978, Goodfellow & Wayne, 1982). Thus, mycobacteria are generally considered to be strongly acid-alcohol fast actinomycetes which produce straight rods, or occasionally branched filaments, whereas nocardiae are seen as weakly acid-alcohol fast organismes that form a substrate and aerial mycelium which undergoes fragmentation into rods and cocci. Not all nocardiae, however, are acid-alcohol fast nor do they always form aerial hyphae (Goodfellow, 1971; Williams *et al.*, 1976) while in a population of mycobacteria the ratio between acid- and non-acid alcohol fast bacilli may be variable (David, 1976). Further, mycobacteria such as *M. farcinogenes* and *M. senegalense* form extensive substrate mycelium (Chamoiseau, 1979) whereas others like *M. xenopi* may bear short aerial hyphae (Runyon, 1968). In contrast to staining and morphological characters, chemical markers have been shown to be of value in both the classification and identification of nocardioform genera (Goodfellow & Schaal, 1979; Minnikin & Goodfellow, 1980).

The walls of Mycobacterium and Nocardia strains contain major amounts of *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV,

Lechevalier & Lechevalier, 1970) but these organisms can be distinguished on the basis of lipid composition (Minnikin & Goodfellow, 1980). In particular, mycobacteria and nocardiae contain different types of mycolic acids, long-chain 2-alkyl-branched 3-hydroxy acids found only in actinomycetes with a wall chemotype IV, which can be distinguished by one of a number of chemical methods. Thus, mycolic acids from strains of *Mycobacterium*, examined to date, produce multispot patterns on thin-layer chromatographic analysis of whole-organism methanolysates whereas those from *Nocardia*, *Rhodococcus*, *Corynebacterium sensu stricto* form a single spot (Minnikin, Alshamaony & Goodfellow, 1975; Goodfellow, Collins & Minnikin, 1976; Minnikin *et al.*, 1980). Mycolic acids from mycobacteria can also be distinguished from those of other actinomycetes by pyrolysis gas chromatography and mass spectrometry of their methyl esters (Etémadi, 1967a; Lechevalier, Horan & Lechevalier, 1971). Use of both procedures leads to the release and identification of long-chain esters having 22 to 26 carbons for mycobacterial mycolates and 12 to 22 carbons for the mycolates of other taxa (Minnikin & Goodfellow, 1980). Again, mycolates from mycobacteria are generally larger (60-90 carbons) than those (20-70 carbons) occurring in related bacteria and mass spectrometry allows such differences to be observed.

Mycobacteria and nocardiae occur in a wide range of natural habitats in Zaire (Portaels, 1973, 1976). In subsequent studies (Portaels, 1978; 1980) 956 mycobacteria and 93 nocardiae were identified to the species level using conventional tests (Pattyn & Portaels, 1972; Portaels, 1976) which were not sufficiently discriminatory to identity 40 slowly growing actinomycetes which had properties lying between those expected for *Mycobacterium* and *Nocardia*. The isolates were strongly acid-alcohol fast, produced a primary mycelium carrying grossly visible aerial hyphae and did not grow or grew very poorly at 37 °C. In the present study most of the isolates were found to form a hitherto undescribed group of mycobacteria.

2. Material and methods

2.1. *Sampling and isolation of strains.* Samples of soil, mud, grass, water and fish were collected from two regions of Zaire separated by approximately 1.500 km. Three hundred and seventy three samples were taken from the savanna in the Kivu region and 1.339 from savana, forest, arable and coastal habitats in Lower Zaire. Samples were pretreated prior to decontamination (Portaels, 1973).

Treated samples were divided into two; one half were decontaminated using the trisodium phosphate methods (Corper and Stoner, 1946) and the other by the oxalic acid procedure (Beerwerth and Schurmann, 1969). Each decontaminated sample was used to inoculate four Lowenstein-Jensen slopes, two of which were incubated at 28 °C and two at 37 °C. The slopes were examined weekly for one year and colonies found to be strongly acid-fast (Portaels, 1978) inoculated onto fresh slopes which were incubated at the same temperature as the primary culture. The origin, method, temperature and time of isolation of the 40 strains obtained are shown in Table 1.

TABLE 1

Source, method, temperature and time of isolation of the test strains

Laboratory number	Source	Method of isolation	Days	Primary isolation temperature (°C)
157	mud, savanna, L.Z. [1]	O[2]	116	28
239	soil, savanna, L.Z.	P[3]	266	28
345	water, savanna, L.Z.	O	61	28
473	water, savanna, L.Z.	P	91	28
474	mud, savanna, L.Z.	O	103	28
483	water, savanna, L.Z.	O	105	28
598	water, savanna, L.Z.	P	83	28
611	water, savanna, L.Z.	O	216	28
629	water, savanna, L.Z.	O	258	28
631	fish, savanna, L.Z.	O	353	28
686	soil, savanna, L.Z.	O	25	28
719	water, savanna, L.Z.	O	273	28
867	water, savanna, Kivu	P	148	28
868	water, savanna, Kivu	O	153	28
869	mud, forest, L.Z.	O	154	28
874	plant, savanna, Kivu	P	137	28
881	fish, savanna, Kivu	P	79	28
888	water, savanna, Kivu	O	121	28
898	water, savanna, Kivu	O	204	28
901	water, savanna, Kivu	O	77	28
996	fish, savanna, L.Z.	P	48	28
1027	coastal sand, L.Z.	O	66	28
1047	water, forest, L.Z.	P	41	37
1086	water, savanna, Kivu	O	215	28
1100	fish, savanna, L.Z.	O	96	28
1120	water, forest, L.Z.	P	124	28
1151	water, coast, L.Z.	O	36	28 and 37
1156	sand, savanna, L.Z.	O	73	28 and 37
1168	fish, savanna, L.Z.	O	48	28 and 37
1183	fish, savanna, L.Z.	P	110	28
1187	fish, savanna, L.Z.	O	105	37
1188	fish, savanna, L.Z.	O	105	28
1192	fish, savanna, L.Z.	O	53	28
1194	fish, savanna, L.Z.	O	104	28
1218	water, savanna, Kivu	P	242	28
1220	soil, forest, L.Z.	O	143	28
1254	fish, savanna, L.Z.	P	147	28
1312	fish, savanna, L.Z.	O	40	28
1319	fish, savanna, L.Z.	O	57	28
1340	fish, savanna, L.Z.	O	196	28

[1] L.Z. : Lower Zaire.
[2] O : oxalic acid method (Beerwerth & Schurmann, 1969).
[3] P : trisodium phosphate method (Corper & Stoner, 1946).

2.2. *Micromorphology, staining and cultural properties.* Smears from cultures grown for one week to one month on Lowenstein-Jensen slopes were prepared, Ziehl-Neelsen-stained, Gram-stained and examined for micromorphological properties. The test strains were also inoculated onto Lowenstein-Jensen, modified Sauton's (Mordarska, Mordarski and Goodfellow, 1972) and glucose yeast extract agar (Gordon and Mihm, 1962) slopes and incubated for 60 days at 28 °C, 33 °C and 37 °C in air as well as in 5 per cent, v/v CO_2 enriched atmosphere (Boncyk, Millstein and Kalter, 1976). The cultures were also inoculated onto oleic acid and modified

Sauton's agar plates and incubated at 28 °C and 33 °C in air. Both slopes and plates were examined weekly when the amount of growth and colony characteristics were recorded.

2.3. *Lipid analysis.* The forty isolates (Table 2) were grown in flasks containing either 0.2 or 2 litres of 7H9 broth (Difco), or in TB broth (Difco) supplemented with calf serum (10 per cent, v/v) and glucose (0.75 per cent, v/v), with continuous magnetic stirring. Cultures were incubated for 2 months at their optimal temperature, namely 28 or 33 °C, when they were checked for purity, killed with formalin 1 per cent, v/v), harvested by centrifugation, washed 3 times with distilled water then freeze dried.

In initial studies, dried biomass was examined using the acid methanolysis and one-dimensional thin-layer chromatography procedure of Minnikin *et al.* (1975). To obtain more precise data on the overall mycolic acid pattern an updated procedure (Minnikin *et al.*, 1980) involving two-dimensional thin-layer chromatography was also applied to certain samples. Mycolic acid methyl esters were isolated from whole-organism methanolysates by preparative thin-layer chromatography on 1 mm layers of Merck silica gel $PF_{254 + 356}$ using a triple development with petroleum ether (b.p. 60-80°)-acetone (95 : 5, v/v). Mass spectra of mycolic acid methyl esters were recorded using an A.E.I. MS9 mass spectrometer using a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 180 to 240 °C.

2.4. *Biochemical and physiological properties.* The mycobacterial cultures were identified by the tests described in the identification scheme of Pattyn and Portaels (1972) and its more recent adapted version (Jenkins *et al.*, 1982).

2.5. *Pathogenicity tests.* Mouse foot pads were inoculated with 0.03 ml of a suspension (1 mg/ml) of each isolate in order to compare the organisms with *Mycobacterium ulcerans* (Pattyn & Rooyackers, 1965).

3. **Results**

The 40 strains studied are slowly growing actinomycetes which can be divided into 4 groups on the basis of their mycolic acids content and their cultural, biochemical and physiological properties (Tables 2 and 3).

3.1. *Micromorphology, staining and cultural properties.* All of the strains were Gram-positive, strongly acid-alcohol fast, grew at 28 and 33 °C but poorly if at all at 37 °C (Table 2). The strains produced short to long branched filaments but the micromorphological properties were influenced by the growth environment, the age of the culture and the time since primary isolation. Colonies were rough and non-pigmented (except for 1168 and 996) and exhibited sparse to abundant aerial hyphae and filamentous margins on oleic acid agar (Figs 1 & 2). Some strains grew more rapidly and produced more aerial mycelium on modified Sauton's medium than on oleic acid agar or Lowenstein-Jensen's medium (group C); only strain 1168 grew well on glucose yeast extract agar. Growth was only slightly enhanced in the CO_2 enriched atmosphere.

TABLE 2

Mycolic acid analysis, morphological and cultural properties of nocardia-like actinomycetes isolated in Zaire

		M.A. (1)		A.H. (2)	Pigment	Growth at	
		> 1 spot	1 spot			28 °C	33 °C
Group A	1168	O	O	–	scoto	+	+
	1218	O	O	–	–	+	±
Group B	686		+	–	–	+	+
	996		+	–	photo	+	+
	1047		+	–	–	+	+
	1120		+	–	–	+	+
Group C	598	+ *		+	–	+	+
	611	+		+	–	+	+
	629	+		+	–	+	+
	719	+		+	–	+	+
	867	+		+	–	+	+
	868	+		+	–	+	+
	898	+ *		+	–	+	+
	1027	+		+	–	+	+
	1100	+ *		+	–	+	+
	1151	+		+	–	+	+
	1183	+		+	–	+	+
	1187	+		+	–	+	+
	1192	+		+	–	+	+
	1312	+		+	–	+	+
	1254	+		+	–	+	+
	1312	+		+	–	+	+
	1319	+ *		+	–	+	+
Group D	157	+		–	–	+	±
	239	+ *		–	–	+	±
	345	+		–	–	+	±
	473	+		–	–	+	+
	474	+		–	–	+	+
	483	+ *		–	–	+	±
	631	+		–	–	+	±
	869	+ *		–	–	+	+
	874	+		–	–	+	±
	881	+		–	–	+	+
	888	+ *		–	–	+	+
	900	+		–	–	+	+
	1086	+ *		–	–	+	–
	1156	+		–	–	+	+
	1194	+		–	–	+	+
	1220	+		–	–	+	+
	1340	+ *		–	–	+	+

(1) M.A. : Mycolic acids patterns determined by single dimensional thin-layer chromatography (t.l.c.) and confirmed for representative strains (asterisk *) by two dimensional t.l.c.
(2) A.H. : Aerial hyphae produced on modified Sauton's medium.

3.2. *Mycolic acid analysis.* Mycolic esters were detected by thin-layer chromatography (t.l.c.) of whole-organism methanolysates in all but two (group A, table 2) of the strains examined. Four of the isolates gave a single mycolic acide ester spot (group B) with a mobility similar to that of Nocardia strains. The 34 remaining strains (group C and D) gave a multi-spot pattern characteristic of mycobacteria, as shown in Fig. 3. In the two-dimensional t.l.c. analysis all 10 strains (Table 2) gave a characteristic pattern, an example of which is shown in Fig. 4. The patterns of mycolates are essentially the same as those observed for strains of *M. avium*, *M. intracellulare*, *M. paratuberculosis* and *M. phlei* (Minnikin *et al.*, 1980) and contain components probably corresponding to 2-eicosanol, ω-carboxymycolates, ketomycolates and α-mycolates, the latter having no other oxygen functions in addition to the 3-hydroxy ester unit (Etémadi, 1967b). In all cases, however, only very low proportions of ketomycolate were observed.

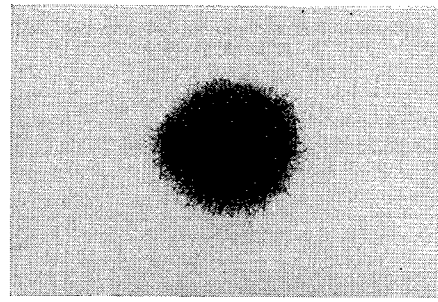

Figure 1.
Colony morphology of strain nº 631 on oleic acid agar medium after 60 days incubation at 28 °C (10 × 10).

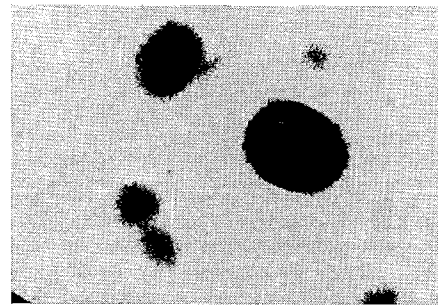

Figure 2.
Colony morphology of strain nº 868 on oleic acid agar medium after 30 days incubation at 33 °C (10 × 10).

Mass spectrometry of the α-mycolates isolated from strains 239 and 1100 showed the presence of peaks at *m/e* 354, *382* and 340 (main component in italics) corresponding to C_{22}, C_{24} and C_{26} straight-chain esters released on pyrolysis (Etémadi, 1967a); the α-mycolate from strain 1187 had a strong peak at *m/e* 382 accompanied by a lesser peak at *m/e* 354. All three α-mycolates contained homologous series of peaks at *m/e* 726, 740, 754, 768, 782, 796, 810 and 824 corresponding to homologous meroaldehydes having from 51 to 58 carbons which were also released by pyrolysis in the mass spectrometer (Etémadi, 1967a). The main meroaldehyde components in strains 1100 and 1187 were at *m/e* 754 and at *m/e* 796 for strain 239. Taking the data for the straight-chain esters and meroaldehydes together, the α-mycolates under study comprise a homologous series (C_{75} to C_{82}) of diunsaturated mycolic acids the main components having 77 carbons for strains 1100 and 1187 and 80 carbons for strain 239.

The mass spectrum of the single mycolic ester isolated from strain 1120 showed a strong peak at *m/e* 242 and a lesser one at *m/e* 270 corresponding to the characteristic release of C_{14} and C_{16} straight chain esters (Etémadi, 1967a). Series of peaks at *m/e* 542, 570, *598*, 626 and *m/e* 596, 624, 652 and 680 correspond to C_{38}, C_{40}, C_{42}, C_{44} triunsaturated aldehydes and C_{42}, C_{44}, C_{46}, C_{48} tetraunsaturated aldehydes, respectively. Small peaks between

m/e 738 and 904 correspond to anhydromycolates having between 50 and 62 carbon atoms. The data indicate that the main components of the mycolic acids of this type have been isolated from nocardiae and rhodococci (Minnikin and Goodfellow, 1980).

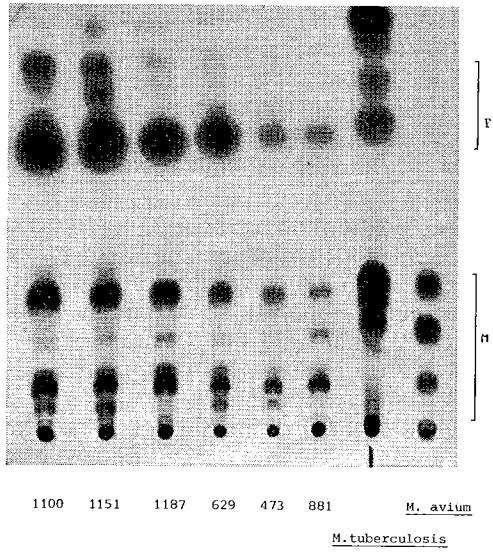

Figure 3.

Single dimensional thin-layer chromatography of whole-organism acid methanolysates of representative test strains and standard strains of *Mycobacterium avium* and *Mycobacterium tuberculosis*. The developing solvent was petroleum ether (b.p. 60-80 °C) — diethyl ether (85 : 15 v/v). Abbreviations : F, non-hydroxylated fatty acid methyl esters; M, mycolic acid methyl esters and related compounds.

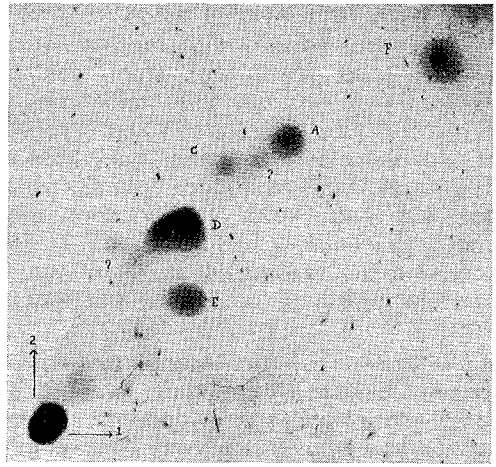

Figure 4.

Two dimensional thin-layer chromatography of the whole-organism acid methanolysate of strain 1187. A triple development with petroleum ether (b.p. 60-80 °C) — acetone (95 : 5 v/v) was used in the first direction followed by a single pass with toluene-acetone (97 : 3 v/v) in the second direction. Abbreviations : F, non-hydroxylated fatty acid methyl esters; A, α-mycolate; C, ketomycolate; D, ω-carboxymycolate; E, 2-eicosanol and homologous alcohols; ?, unknown minor components.

3.3. *Biochemical and physiological properties.* The 34 isolates giving a multispot pattern characteristic of mycobacteria were divided in 2 groups, (C and D), on the basis of cultural, biochemical and physiological properties (Table 3). The table also contains data on three other slowly growing mycobacteria : *M. shimoïdei*, *M. xenopi* and *M. avium*. Although *M. xenopi* (Runyon, 1968) and *M. shimoïdei* (Wayne *et al.*, 1981) produce non-pigmented rough colonies, covered with sparse aerial hyphae, on oleic acid albumin agar, the mycobacterial strains of groups C and D differ from them by more two characters (Table 3). Further, while the patterns of mycolates of strains in groups C and D resemble those of *M. avium* they differ from the latter in some biochemical and physiological properties (Table 3).

A number of characters separate groups C and D (Table 3) : resistance to para-nitrobenzoic acid and hydroxylamine, acid phosphatase, benzamidase (amide 2) activity and production of an abundant aerial mycelium on modified Sauton's medium. Moreover some strains belonging to group D grow very poorly at 33 °C whereas group C strains grew optimally at 33 °C (Tables 2 and 3).

3.4. *Pathogenicity tests.* None of the 40 test strains were pathogenic for mice.

4. **Discussion**

Mycobacterial mycolic acids, possibly the most characteristic component of the mycobacterial cell, are recognised by their structural complexity, high molecular weight (C_{60} to C_{90}), formation of C_{22}, C_{24} and C_{26} straight chain esters on pyrolysis, and their ability to form a multispot pattern on t.l.c. of whole-organism methanolysates (Minnikin & Goodfellow, 1980; Minnikin *et al.*, 1980). The mycolic acids from 34 of the 40 strains examined formed a multispot pattern with representative organisms producing long-chain α-mycolates (C_{75} to C_{82}), which yielded C_{22}, C_{24} and C_{26} straight chain esters on pyrolysis gas chromatography, and components which probably correspond to ω-carboxy-and keto-myolates. These properties are consistent with the classification of the 34 isolates in the genus *Mycobacterium*. The organisms produced colonies similar to those of *M. xenopi* (Runyon, 1968) and like *M. farcinogenes* and *M. senegalense* they produce a primary mycelium (Chamoiseau, 1979). Further work needs to be done to determine the taxonomic status of the four isolates that produced mycolic acids typical of nocardiae and the two strains which lacked mycolic acids.

The mycobacteria formed two homogenous groups on the basis of morphological, biochemical and physiological properties (Table 2 and 3) with representatives giving a pattern of mycolates reminiscent of *M. avium*, *M. intracellulare*, *M. paratuberculosis* and *M. phlei* (Minnikin *et al.*, 1980). Although the isolates do share properties with other non-pigmented, slow growing mycobacteria they differ from all other mycobacteria known at present on the basis of the characters listed in table 3. They can also readily be distinguished from *M. ulcerans* as they do not cause local swellings or ulceration when inoculated into footpads of mice.

TABLE 3

Characters useful for differentiating among Nocardia-like mycobacteria

	Scotochromogenic	Nonchromogenic	Growth at (°C) 33	37	42	Resistance to I	P	T	H	Catalase > 45 mm	Niacin	Nitratase	Acid phosphatase	Amidases 2	3	5	6	A.H.	Colony on OAA
Group C	—	+	+	—	—	—	+	+	+	—	—	—	—	+	—	+	+	+	Noc
Group D	—	+	M	—	—	F	—	+	—	—	—	—	+	—	—	—	—	—	Noc
M. xenopi	+	—	+	+	+	—	+	+	M	—	—	—	—	—	—	+	+	—	Noc
M. shimoidei	—	+	+	+	+	—	+	+	—	—	—	—	+	—	—	+	+	—	Noc
M. avium	—	+	+	+	+	M	+	+	+	—	—	—	—	—	—	+	+	—	SmS-SmT

\+ = > 85 % of strains pos.
— = < 15 % of strains pos.
M = 50 to 85 % of strains pos.
F = 15 to 49 % of strains pos.
OAA = oleic acid albumin agar.
A.H. = aerial hyphae on modified Sauton's medium.

Noc = Nocardia-like colonies with aerial hyphae.
SmS = Smooth — Scotochromogenic.
SmT = Smooth — Transparent.
I = isoniazid 10 μg/ml.
P = p-nitrobenzoïc acid 500 μg/ml.
T = thiophene-2-carboxylic acid hydrazide 1 μg/ml.
H = hydroxylamine hydrochloride 250 μg/ml.

New species of both fast and slow growing mycobacteria have been proposed to accommodate mycobacteria from a diverse range of natural habitats but some of these have been reduced to synonyms of established species (Kubica *et al.*, 1972; Runyon, Wayne & Kubica, 1974) whereas others were considered to be insufficiently described to merit inclusion in the Approved Lists of Bacterial Names (Skerman, McGowan & Sneath, 1980). Thus, while the isolates from Zaire appear to form two new taxa, additional comparative studies are required to determine their detailed relationships with other slow growing mycobacteria.

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Un nouveau groupe de mycobacteries isolées de l'environnement au Zaïre.

Résumé — Quarante Actinomycétales à croissance lente ont été isolées de l'environnement au Zaïre à partir de divers biotopes. Il s'agit de bacilles acido-alcoolo-résistants formant des colonies recouvertes d'un abondant mycélium aérien. Parmi ces bactéries, 34 souches possèdent des acides mycoliques caractéristiques des mycobactéries et se subdivisent en deux groupes homogènes d'après leurs propriétés morphologiques, physiologiques et biochimiques. Ces deux nouveaux groupes de mycobactéries diffèrent de toutes les espèces de mycobatéries connues actuellement.

Nocardia-achtige mycobacteriën geïsoleerd uit de omgeving in Zaïre.

Samenvatting — Uit de omgeving in Zaïre werden veertig traaggroeiende stammen Actinomycetales geïsoleerd van verschillende biotopes. Het betreft zure-alkohol-weerstandige bacillen, die kolonies vormen met overvloedige luchthyfen. Vierendertig van deze bacteriënstammen bevatten mycolzuren, karakteristiek voor mycobacteriën en kunnen volgens hun morfologische, fysiologische en biochemische eigenschappen, onderverdeeld worden in twee homogene groepen. Deze twee nieuwe groepen mycobacteriën verschillen van alle tot nu bekende soorten mycobacteriën.

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