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Lipid Composition in the Classification of Nocardiae and Mycobacteria

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Ninety-six strains of aerobic actinomycetes with a type IV cell wall (major amounts of meso-diaminopimelic acid, arabinose, and galactose) were analyzed for the presence of mycolic acids and nocardomycolic acids. The method used was comparatively simple and permits the separation of these organisms into two groups: the mycobacteria and the nocardiae. In general, strains received as mycobacteria contained mycolic acids, confirming the generic assignment made by other methods. On the basis of nocardomycolic acid content, *Mycobacterium brevicale*, *M. rhodochrous*, and *M. thamnopheos* should be placed in the genus *Nocardia*, and on the basis of mycolic acid content, strains recently isolated from bovine farcy should be placed in the genus *Mycobacterium*. *Nocardia farcinica* should be considered a *nomen dubium* and *N. asteroides* should be considered the type species of the genus.

The generic name *Nocardia* has been used in the past to refer, somewhat loosely, to filamentous, branching, aerobic bacteria that do not seem to fall squarely into any of the better-defined genera of the *Actinomycetales* (22). The type species of the genus is *N. farcinica*, first isolated and described by Nocard in 1888 (26), and named a year later by Trevisan (32). Nocard's description is vague but refers to an aerobic organism growing easily on laboratory media. Reference is made to the "dusty," nonwettable surface of the growth on some media and to the abundant production of spores. Considering (i) the vagueness of Nocard's description (ii) the absence in any culture collection of an authentic strain representing Nocard's original isolate, and (iii) the rarity of references to *N. farcinica* in the literature, Gordon and Mihm (15) proposed that *N. asteroides*, described by Eppinger in 1891 (12), should be considered the type species of the genus.

Pending further studies on the nature of *N. farcinica*, we decided to consider *N. asteroides*, at least tentatively, as the type species of the genus. Our approach has been to study all organisms that could conceivably be placed in the genus *Nocardia* and to remove systematically from this mass of strains, groups of organisms that could clearly be separated by major characteristics from *N. asteroides*.

Following the lead of Cummins (11), we proposed that actinomycetes containing major amounts of L-2,6-diaminopimelic acid (DAP) should not be placed in the genus *Nocardia*. This permitted us to eliminate from the nocardiae all streptomycetes (5, 28) which, because they lacked aerial mycelium or had a "soft" growth, could not be otherwise distinguished from this group. Further studies of the cell wall composition of aerobic actinomycetes showed that nocardiae could be separated into two broad groups (24): (i) those with mureins containing meso-DAP, glucosamine, muramic acid, alanine, and glutamic acid (*madurae*-type); and (ii) those whose cell wall preparations are, in addition, rich in arabinose and galactose (*asteroides*-type).

The genus *Actinomadura* was proposed to harbor nocardiae of the *madurae* type. These were divided, on the basis of whole-cell sugar pattern and morphology, into two subgroups: (i) the actinomadurae of the *madurae* type, which includes *pelletieri*, and (ii) those of the *dassonvillei* type (23). The genus *Oerskovia* was proposed (27) to harbor organisms previously called *N. turbata*. These motile organisms are characterized, among other things, by a cell wall lacking major amounts of DAP, a property that sharply differentiates them from the nocardiae of the *asteroides* type. Oerskoviae can also be distinguished from members of other genera by phage typing (H. Prauser, Recent Progr. Microbiol., *in press*). The erection of these new genera is supported not only by these data but also by results obtained in numerical taxonomic studies

(M. Goodfellow, Recent Progr. Microbiol. *in press*).

Work on cell wall composition and morphology has been unsuccessful in permitting a separation between nocardiae of the *asteroides* type, mycobacteria, and some corynebacteria. All have a type IV cell wall (6) and their morphology presents an uninterrupted spectrum from mucoid bacteria with a shade of branching to highly filamentous, highly branched organisms bearing chains of conidia. Previous work by others which has been summarized by Asselineau (3) and Lanéelle-Carrieu (Thesis, Toulouse, 1969) has shown that strains of the *Nocardia-Mycobacterium-Corynebacterium* complex having a type IV cell wall composition contain α-branched, β-hydroxylated fatty acids, called mycolic acids, which are closely associated with the cell wall. Mycolic acids seem to fall into three groups: (i) those with carbon skeletons of about 80 carbon atoms associated with strains of *Mycobacterium* (mycolic acids *stricto sensu*), (ii) those with skeletons of about 50 carbons found in nocardiae (nocardomycolic acids), and (iii) those with smaller skeletons of about 30 atoms of carbon which seem to be the appanage of corynebacteria (corynomycolic acids).

We are reporting here a comparatively simple method for the detection of the types of mycolic acids present in a given actinomycete and the present state of our continuing survey of the lipids present in nocardiae and mycobacteria. These data are of importance since the results previously reported by other authors were based on the examination of very few strains, and sound taxonomic conclusions could not be drawn.

MATERIALS AND METHODS

Organisms. The organisms included in this study are listed in Table 1. Strains growing at 28 C were incubated on a reciprocal shaker (New Brunswick Scientific Co., New Brunswick, N.J.; model 5713, operated at 60 strokes/min) for 1 or 2 weeks. The growth medium was either YD broth (yeast extract-glucose broth; reference 33) or Bennett's broth (17). Strains growing at 37 C were incubated statically for 4 to 5 weeks. The medium employed was either Bennett's broth, Sauton's (3), modified Sauton's (substituting equimolar amounts of formate or acetate for citrate), or TB Broth Base (Difco) without Tween 80 or serum. Not all organisms would grow under the same conditions. Nevertheless, 85% of the strains studied were grown under standard conditions, namely, on YD broth incubated on a shaker for 1 week. After incubation, the cultures were autoclaved and harvested by filtration or centrifugation.

Preparation of mycolates. The moist biomass thus obtained was placed in a 500-ml Erlenmeyer flask, and enough 95% ethanol was added to cover the cells. The stoppered flask was placed on the reciprocal shaker for 1 week. The cells and solvent were separated by gravity filtration, and the biomass was reextracted with methylene chloride for an additional week. The methylene chloride extract was taken to dryness at room temperature (25 C) in a stream of air, and 200 to 300 mg of the resulting residue was placed in a tube (13 by 100 mm) to which 5.0 ml of 6% methanolic KOH was added. Saponification took place overnight in a 60 C oven. The methanol was evaporated under reduced pressure at 45 to 50 C. To the residue, 5.0 ml each of methylene chloride and distilled water was added, and the whole was shaken thoroughly in a tube (20 by 150 mm) with a vibrator-mixer. The resulting emulsion was broken by centrifugation at 1,200 $\times$ *g*, and the sample was divided into an aqueous (A) and an organic-soluble (O) fraction. Five milliliters of water was added to the O fraction and the same amount of methylene chloride was added to the A fraction. These were shaken, centrifuged as before, and divided into A and O fractions; the like fractions from this and the previous separation were combined. Methylene chloride was added to the A fraction (1:2), distilled water was added to the O fraction (1:2), and both fractions were acidified with 6 N HCl to a *p*H of 2.0. Each fraction was mixed and centrifuged, and the acidified aqueous phase was discarded. The methylene chloride from both fractions was evaporated under reduced pressure at 45 to 50 C. Final drying was carried out in a vacuum oven with concentrated H_2SO_4 used as desiccant. The dried residues were methylated by using a 10% (w/v) solution of boron trichloride in methanol (Applied Science Laboratories, State College, Pa.), by the method of Metcalfe and Schmitz (25), substituting methylene chloride for petroleum ether. Solid materials formed at the interface were retained with the organic phase, as they were often found to be rich in mycolates. Up to 60 mg of the resulting residue was purified by preparative thin-layer chromatography on a single plate [4 by 8 inches (10 by 20 cm)] coated with Merck Silica Gel PF_{254} (Brinkmann Instruments, Inc., Westbury, N.Y.) developed with petroleum ether (boiling point 40 to 50 C)-diethyl ether (8:2). After air-drying, the lipid-containing bands were revealed by spraying with Rhodamine B (20). Compounds migrating the same distance as a pure sample of mycolic acid ester were scraped from the plate and eluted with methylene chloride. Four to 10 μg of each dried eluate redissolved in methylene chloride was injected into a Perkin-Elmer model 810 dual-column gas chromatograph equipped with a hydrogen flame detector and stainless-steel columns [6 ft by 1/8 inch ($\sim$1.8 m by 0.32 cm)] packed with Chromosorb W, 80 to 100 mesh (AW-DMCS) with 3% OV-1 as liquid phase. Conditions for analysis were: detector, 270 C; injector, 300 C; oven, 185 C (for fatty esters) or 170 C (for fatty aldehydes); programmed to 270 C at 4 C/min. The carrier gas was nitrogen flowing at 30 ml/min.

Pyrolysis fragments were identified by their retention times. Used as standards were even, saturated, straight-chain methyl esters of fatty acids of carbon chain lengths 12 through 26 and the corresponding fatty aldehydes through C_{18} which were stabilized in CS_2 (Applied Science Laboratories). Methyl esters of pure mycolic and nocardomycolic acids were generously supplied by J. Asselineau.

RESULTS AND DISCUSSION

Pyrolysis of the mycolic esters in the gas chromatograph permits the separation of the mycobacterial mycolates from the nocardomycolates and the corynomycolates because, under these conditions, the former molecules split to release unbranched saturated fatty esters having 22 to 26 carbons, whereas, from the two latter types, C_{12} to C_{18} fatty esters are released.

A distinction can be made between corynomycolates and nocardomycolates on the basis of the aldehyde moiety formed during pyrolysis. The aldehydes from nocardomycolates, because of their large size ($C_{35} \pm 3$ C), are retained on the column of the gas chromatograph under the conditions we use, whereas those from corynomycolates, being smaller (C_{16} to C_{18}), are eluted and can be identified by their retention times.

Classical procedures for the extraction of mycolic acids call for the preliminary extraction of cells with an ethanol-ether mixture followed by extraction with chloroform (3). Nocardo- and corynomycolic acids were found in the first extract, mycolic acids in the second. We have observed that methylene chloride extracts all three types of mycolic acids from the cells and since, unlike ether, its vapors are non-flammable and not explosive when mixed with air, its use presents no hazard in microbiological laboratories where open flames are common.

One of us (A. C. Horan, Ph.D. thesis, Rutgers Univ., 1970) demonstrated that there were no qualitative differences between the mycolic acids formed by *M. smegmatis* A 607 and *N. asteroides* A 3318 when grown on two different media in static and shaken cultures. These same two organisms and *N. farcinica* N 4524 were also grown in shaken cultures for various lengths of time up to 24 days. The production of mycolic acids was found to increase with the age of the culture but, for qualitative analyses, it was found that cells 1 week old or more contain enough of these acids for easy detection. These results would indicate that changes in nutritive and growth conditions can affect the amounts of mycolic acids produced but seem to have no effect on the types of mycolic acids formed.

In Table 1 are listed strains of *Nocardia* and *Mycobacterium* which we analyzed for mycolic acids. All have cell walls of type IV.

Except for strains of *M. brevicale*, *M. rhodochrous*, the so-called Tarshis variants of *M. tuberculosis* and *M. thamnopheos*, all strains received as mycobacteria contained mycobacterial mycolic acids.

M. brevicale was described by Krassilnikov in 1941 (18). According to Krassilnikov (19), *M. brevicale* is closely related to *M. rubrum* and *M. equi* (*Corynebacterium equi*). Gordon (13) found that *rubrum* and *equi* were synonymous with *M. rhodochrous*, which in turn was a species "in search of a genus." It is thus not surprising that we found *M. brevicale* to contain nocardomycolic acids, as do all strains of *rhodochrous* so far studied. While checking the history of the name *M. rubrum*, we noted that Buchanan et al. (7) erroneously stated that Krassilnikov described, in 1949, an organism under the name of *M. rubrum* which was different from the *M. rubrum* of Söhngen (29). We have found no indication that Krassilnikov proposed a new taxon with such a name either in his 1949 or in his 1941 book. Crowle (10), however, proposed as a new taxon, *Corynebacterium rubrum*. Gordon (13) considered it to be also a synonym of *rhodochrous*. Her decision is confirmed by our analyses since strain I 1240, which is Crowle's organism, contains nocardomycolic acids as do the other strains of *rhodochrous*.

M. rhodochrous has been the object of a thorough study by Gordon (13), who demonstrated an extensive synonymy and a lack of clear-cut generic affinity. Falling into the *rhodochrous* group are members of the *M. rubrum* and *Proactinomyces ruber* groups of Krassilnikov (18, 19), *Jensenia canicruria* (14), and numerous so-called mycobacteria, corynebacteria, and nocardiae. The strains of *rhodochrous* that we have studied so far contain nocardomycolic acids and we are endorsing, temporarily at least, their taxonomic assignment to the genus *Nocardia*. This was also the conclusion of Lanéelle et al. (21) based on the examination of two strains.

The so-called "Tarshis strains" are derived from a strain labeled "*M. tuberculosis*" SM-CV2, received by R. E. Gordon from M. Tarshis in 1959. Strain SM-CV2 was a chromogenic variant of *M. tuberculosis* H37Rv grown on media containing streptomycin (31). A single-colony isolate of SM-CV2, which was orange and rough, received IMRU number 1071 (see footnote *b*, Table 1). After repeated transfers and plating out of 1071, three single colony isolates were obtained: 1071W-white; 1071C-deep orange, rough; and, 1071A-orange, rough (parental type). Gordon compared the white strain to the parent and found them to have identical physiological properties. All these strains contain nocardomycolic acids and although we have not studied the parent strain of H37Rv used by Tarshis, we assume that it contained true mycolic acids. Mycolic acids are associated with the cell wall (1), and we found that cell wall type does not change under the influence of mutagenic agents (30). These results suggest that more studies on the effect of muta-

TABLE 1. *Generic assignment of some actinomycetes with type IV[a] cell wall on the basis of pyrolysis of mycolic acids produced*

Culture received as	Culture no.[b]	Major fatty ester fragments released on pyrolysis	Generic assignment
Nocardia asteroides	A 3318	C_{14}, C_{16}	*Nocardia*
N. asteroides	D 3418	C_{16}	*Nocardia*
N. asteroides	E 9991	C_{14}, C_{16}	*Nocardia*
N. asteroides	I 3409B	C_{12}, C_{14}, C_{16}	*Nocardia*
N. asteroides	I 539	C_{14}, C_{16}	*Nocardia*
N. asteroides	N 659	C_{16}	*Nocardia*
N. asteroides	I 433N	C_{14}, C_{16}	*Nocardia*
N. asteroides	LL-5000S	C_{14}, C_{16}, C_{18}	*Nocardia*
N. asteroides	I 727	C_{16}, C_{18}	*Nocardia*
N. asteroides	LL-Z16	C_{14}, C_{16}, C_{18}	*Nocardia*
N. asteroides	I 611	C_{14}, C_{16}	*Nocardia*
N. brasiliensis	I 774B	C_{16}	*Nocardia*
N. brasiliensis	I 800	C_{14}, C_{16}, C_{18}	*Nocardia*
N. brasiliensis	I 849	C_{14}, C_{16}	*Nocardia*
N. brasiliensis	I 556	C_{16}, C_{18}	*Nocardia*
N. brasiliensis	I 1093	C_{14}, C_{16}, C_{18}	*Nocardia*
N. brasiliensis	I 1120	C_{16}, C_{18}	*Nocardia*
N. brasiliensis	I 1188	C_{14}, C_{16}, C_{18}	*Nocardia*
N. carneus	I 3419	C_{16}, C_{18}	*Nocardia*
N. caviae	N 1934	C_{16}	*Nocardia*
N. caviae	I 1233	C_{16}	*Nocardia*
N. caviae	I 1259	C_{16}	*Nocardia*
N. caviae	I 1351	C_{14}, C_{16}	*Nocardia*
N. caviae	I 1342	C_{14}, C_{16}, C_{18}	*Nocardia*
N. caviae	I 732	C_{14}, C_{16}, C_{18}	*Nocardia*
N. farcinica	I 1360 (M-396)	C_{24}	*Mycobacterium*
N. farcinica	I 1226 (M-NF5)	C_{22}, C_{24}	*Mycobacterium*
N. farcinica	I 1223 (M-Fa-4)	C_{22}, C_{24}	*Mycobacterium*
N. farcinica	I 1224 (M-Fa-7)	C_{22}, C_{24}	*Mycobacterium*
N. farcinica	I 1363 (M-434C)	C_{24}	*Mycobacterium*
N. farcinica	I 1364 (M-435C)	C_{24}	*Mybocacterium*
N. farcinica	I 1243	C_{22}, C_{24}	*Mycobacterium*
N. farcinica	N 4524	C_{24}	*Mycobacterium*
N. farcinica	I 1361 (M-397)	C_{24}	*Mycobacterium*
N. farcinica	I 1242	C_{24}	*Mycobacterium*
N. farcinica	I 1362 (M-433 C)	C_{24}	*Mycobacterium*
Nocardia sp.	N 2392	C_{16}, C_{18}	*Nocardia*
Nocardia sp.	I 1222	C_{16}, C_{18}	*Nocardia*
Nocardia sp.	I 1105	No mycolic acids	?
Nocardia sp.	I 1320	No mycolic acids	?
Nocardia sp.	I 1124	No mycolic acids	?
Nocardia sp.	I 1337	No mycolic acids	?
Mycobacterium aurum	A 23366	C_{22}, C_{24}	*Mycobacterium*
M. bovis	A 11621	C_{24}, C_{26}	*Mycobacterium*
M. bovis	A 9834	C_{22}, C_{24}, C_{26}	*Mycobacterium*
M. bovis	T503-39	C_{22}, C_{24}, C_{26}	*Mycobacterium*
M. brevicale	A 15113	C_{16}, C_{18}	*Nocardia*
M. chitae	A 19628	C_{16}, C_{22}, C_{24}	*Mycobacterium*
M. diernhoferi	A 19340	C_{22}, C_{24}	*Mycobacterium*
M. flavescens	A 14474	C_{22}, C_{24}, C_{26}	*Mycobacterium*
M. fortuitum	N 8573	C_{24}	*Mycobacterium*
M. fortuitum	I 1000	C_{24}	*Mycobacterium*
M. fortuitum	I 1000BB	C_{24}	*Mycobacterium*
M. fortuitum	LL 2867	C_{22}, C_{24}	*Mycobacterium*
M. fortuitum	I 788	C_{22}, C_{24}	*Mycobacterium*
M. gallinarum	A 19710	C_{24}, C_{26}	*Mycobacterium*
M. gastri	A 15754	C_{22}, C_{24}	*Mycobacterium*
M. intracellulare	I 3600B	C_{24}	*Mycobacterium*
M. kansasii	A 12479	C_{24}	*Mycobacterium*

TABLE 1. *Continued*

Culture received as	Culture no.[b]	Major fatty ester fragments released on pyrolysis	Generic assingment
M. marinum	A 927	C_{24}	*Mycobacterium*
M. novum	A 19619	C_{24}	*Mycobacterium*
M. novum	A 19620	C_{22}, C_{24}	*Mycobacterium*
M. paraffinicum	I 914	C_{22}, C_{24}, C_{26}	*Mycobacterium*
M. parafortuitum	A 19686	C_{22}	*Mycobacterium*
M. phlei	A 37	C_{18}, C_{22}, C_{24}, C_{26}	*Mycobacterium*
M. phlei	A 451	C_{22}, C_{24}	*Mycobacterium*
M. phlei	I 5	C_{22}, C_{24}	*Mycobacterium*
M. phlei	N 8151	C_{22}, C_{24}	*Mycobacterium*
M. phlei	I 8a	C_{22}, C_{24}	*Mycobacterium*
M. rhodochrous	I 1082S	C_{14}, C_{16}	*Nocardia*
M. rhodochrous	I 502	C_{16}	*Nocardia*
M. rhodochrous	I 3407	C_{12}, C_{14}, C_{16}	*Nocardia*
M. rhodochrous	I 764	C_{16}	*Nocardia*
M. rhodochrous	N 1621	C_{16}	*Nocardia*
M. rhodochrous	I 1082R	C_{14}, C_{16}	*Nocardia*
M. rhodochrous	I 545	C_{14}, C_{16}	*Nocardia*
M. rhodochrous	I 1240	C_{16}, C_{18}	*Nocardia*
M. smegmatis	A 607	C_{24}	*Mycobacterium*
M. smegmatis	I 3	C_{22}, C_{24}	*Mycobacterium*
M. smegmatis	I 391	C_{22}, C_{24}	*Mycobacterium*
M. smegmatis	I 455	C_{22}, C_{24}	*Mycobacterium*
M. smegmatis	I 375	C_{22}, C_{24}	*Mycobacterium*
M. smegmatis	I 77	C_{22}, C_{24}	*Mycobacterium*
M. terrae	A 15755	C_{24}, C_{26}	*Mycobacterium*
M. thamnopheos	A 4445	C_{16}	*Nocardia*
M. thermoresistibile	A 19527	C_{24}	*Mycobacterium*
M. tuberculosis	H 37Ra	C_{26}	*Mycobacterium*
M. tuberculosis (Tarshis variant)	I 1071A	C_{16}, C_{18}	*Nocardia*
	I 1071C	C_{16}, C_{18}	*Nocardia*
	I 1071W	C_{16}, C_{18}	*Nocardia*
M. vaccae	I 85	C_{22}, C_{24}	*Mycobacterium*
M. vaccae	I 523A	C_{22}, C_{24}	*Mycobacterium*
M. vaccae	A 23027	C_{22}, C_{24}	*Mycobacterium*
M. vaccae	I 282	C_{22}, C_{24}	*Mycobacterium*
M. vaccae	A 23002	C_{22}, C_{24}	*Mycobacterium*

[a] Preparations of type IV cell wall contain major amounts of *meso*- or D-2,6,-diaminopimelic acid (or both), arabinose, and galactose in addition to major amounts of glucosamine, muramic acid, glutamic acid, and alanine.

[b] A = ATCC (American Type Culture Collection, Rockville, Md.); D = Duke University; E = C. W. Emmons, National Institutes of Health, Bethesda, Md.; I = IMRU (Institute of Microbiology, Rutgers University); LL = authors' collection; N = NCTC (National Collection of Type Cultures, London, England); T = E. H. Titsworth, Hoffman-LaRoche, Nutley, N.J. M = F. Mariat, Institut Pasteur, Paris.

gens and antibiotics on mycolic acid composition would be of interest.

We observed no change in mycolic acid composition in the case of a variant from *M. fortuitum* IMRU 1000. From this organism, which is usually cream-colored, Gordon and Pang (16) isolated a deep blue-black variant, IMRU 1000BB, which still had the physiological properties and the mycolates of its parent. Also, no difference was noted between *M. rhodochrous* 1082R (rough) and 1082S (smooth).

M. thamnopheos was described in 1929 by Aronson (2), who isolated it from a tuberculous lesion of a snake. It lacks true mycolic acids and should be considered a *Nocardia*. More strains of this type will be needed before its taxonomic position can be more precisely evaluated.

Except for strains of *N. farcinica* and certain strains called *Nocardia* spp., all strains received as nocardiae contained nocardomycolic acids. As far as *N. farcinica* is concerned, in a previous publication we reported briefly on the detection of mycobacterial mycolates in 11 strains of organisms from cases of bovine farcy (A. C. Horan, M. P. Lechevalier, and H. Lechevalier, Bacteriol. Proc., p. 20, 1970). This confirmed the results of other investigators (4, 8). We concluded that *N. farcinica* is a *nomen dubium* and that *N. asteroides* should be considered the type species of the genus, as proposed by Gordon and Mihm (15).

In recent communication, Chamoiseau and Asselineau (9) reported the detection of mycolic acids in *N. farcinica* strain 378 of the Pasteur Institute in Paris. They stated that this culture was received by them as an authentic strain of *N. farcinica*. This implies that strain 378 (ATCC 13781) is identical to Nocard's original strain. However, F. Mariat of the Pasteur Institute in Paris has assured us that he knows of no strain that could, with confidence, be considered Nocard's original (*personal communication*). According to S. A. Waksman's records, strain ATCC 3318 is Nocard's original isolate of *N. farcinica*. Although superficially somewhat different, strain NCTC 4524 is supposed to be a duplicate of ATCC 3318. Nevertheless, the former contains mycolic acids and the latter nocardomycolic acids. Hence, no strain can be proved to be "authentic," since no indisputable type strain exists and since Nocard's original description is too vague to be of real use in identification. In addition, the Pasteur Institute culture catalogue indicates that strain 378 comes from Dakar, Senegal, whereas Nocard isolated his original strain from material coming from Guadeloupe, an island now free of the disease (G. Chamoiseau, *personal communication*). The etiology of the strain is not sufficient, in our view, to establish its identity. Hence, we still consider *N. farcinica* to be a *nomen dubium*.

The systematic position of the strains without mycolic acids will require further study.

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