Journal of General Microbiology (1972), **71**, 77–86
Printed in Great Britain

Chemotaxonomic Characters and Classification of Some Nocardioform Bacteria

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(*Accepted for publication* 6 *January* 1972)

SUMMARY

Simple chemical analyses were carried out on 198 nocardioform bacteria by means of paper- and thin-layer chromatography. The strains considered to belong to the genus *Nocardia* contained the lipid LCN-A, arabinose and *meso*-diaminopimelic acid. All the representative strains from the '*Mycobacterium*' *rhodochrous* complex possessed this lipid though in certain cases the characteristic spot had a slightly lower R_F value than that of the reference lipid LCN-A from the standard strain of *Nocardia asteroides*. The genera *Actinomadura*, *Mycobacterium*, *Oerskovia* and *Streptomyces* did not contain lipid LCN-A and the distribution of the other two chemical characters varied. The method used to detect lipid LCN-A is simple and reliable and permits the separation of nocardias and '*M.*' *rhodochrous* strains from allied taxa. These results correlate well with other trends in the taxonomy of nocardioform bacteria and confirm the value of chemotaxonomic characters, especially lipids, in the classification and identification of these organisms.

INTRODUCTION

The term nocardioform was coined by Prauser (1967) to refer to bacteria able to produce a primary mycelium which by regular division gives rise to cocci and rod-shaped elements. The taxonomy of these bacteria is still difficult but, as the results of modern studies accumulate, an outline of some of the major groups is emerging. Groups of nocardioform strains proposed on the basis of wall composition and whole organism hydrolysate analyses (Becker, Lechevalier & Lechevalier, 1965; Lechavalier & Lechevalier, 1970*a*) have been recovered as major clusters in an extensive numerical taxonomic study (Goodfellow, 1971). The chemotaxonomic and phenetic evidence has unequivocally shown that the genus *Nocardia* as characterized in *Bergey's Manual* (Waksman, 1957) is heterogeneous and these data support the case for the acceptance of the newly proposed genera *Actinomadura* (Lechevalier & Lechevalier, 1970*b*) and *Oerskovia* (Prauser, Lechevalier & Lechevalier, 1970). The erection of these taxa leaves the genus *Nocardia* as a homogeneous group containing the major species *Nocardia asteroides*, *N. brasiliensis* and *N. caviae*.

It is not possible, at present, to separate nocardias *sensu novo*, mycobacteria and certain corynebacteria by wall composition characters. The strains in these three genera all have walls of Type IV (Becker *et al.* 1965). Simple but reliable tests for the differentiation of these taxa are required. Additional chemical data may be useful because some chemotaxonomic characters have been shown to be stable under various environmental conditions and even

appear to be unaffected by the age of the culture or by mutagens (Šuput, Lechevalier & Lechevalier, 1967).

Preliminary studies suggest that lipid composition may be useful in the classification of actinomycetes (Lanéelle, Asselineau & Castelnuovo, 1965; Etémadi, 1967*a*, *b*). In an extensive study using pyrolysis gas chromatography, Lechevalier, Horan & Lechevalier (1971) distinguished between *Mycobacterium*, *Nocardia* and *Corynebacterium* by the kind of mycolic acids they possessed. Previous to this Mordarska (1968) and Mordarska & Mordarski (1969) had found a lipid which seemed to be specific for nocardias. This lipid was subsequently called Lipid Characteristic of *Nocardia* or LCN-A (Mordarska & Réthy, 1970). We are reporting here an extension of these studies in which a comprehensive collection of nocardia strains and representatives of the genera *Actinomadura*, *Mycobacterium*, *Oerskovia*, *Streptomyces* and the '*Mycobacterium*' *rhodochrous* complex were examined for the presence of lipid LCN-A, arabinose and diaminopimelic acid (DAP). Sufficient strains were studied for significant conclusions to be drawn on the taxonomic importance of lipid LCN-A.

METHODS

Organisms and growth conditions. The name, number and concise history of the 198 strains tested are listed in Tables 1 and 2. Many of these organisms were included in the numerical study of Goodfellow (1971) where comprehensive strain histories can be found. All the cultures were grown in shake flasks at 37 °C for 3 to 5 days in glucose peptone broth (Prauser & Falta, 1968) and in modified Sauton's medium (Mordarska, 1968). The latter contained (g/l distilled water): glucose, 15·0; asparagine, 5·0; casein hydrolysate, 2·0; sodium citrate, 1·5 g; KH_2PO_4, 5·0; $MgSO_4.7H_2O$, 0·5; K_2SO_4, 0·5; and ferric ammonium citrate, a trace; pH 7·2. The glucose was sterilized separately.

Preparation of lipid extracts. Cultures were checked for purity at maximal growth, killed by shaking with 1 % (v/v) formalin, harvested by filtration and thoroughly washed with water. Two ml of an ethanol–diethyl ether mixture (1:1) was added to 100 mg of dried crushed organism. The centrifuge tubes were sealed with Parafilm (American Can Co., Wisconsin), and left at room temperature for 3 to 4 h. For a good extraction the tubes were occasionally shaken and the solvent mixtures changed twice. The supernatant obtained after centrifugation was evaporated to dryness at 35 C° and the residue stored at 4 °C until use. Lipid extracts from *Nocardia asteroides* USA, *N. calcarea* IMET 7018 and *Streptomyces griseus* 22, which had been examined in previous studies (Mordarska, 1968; Mordarska & Mordarski, 1970) were used as standard references.

Chromatography. Glass plates 20 × 20 cm were covered with a 0·5 mm layer of silica gel G (E. Merck Ag, Darmstadt, W. Germany) and activated for 2 h at 110° C. The lipid residue was dissolved in a 0·05 to 0·1 ml mixture of chloroform and methyl alcohol (3:1), and 25 to 50 μl of this extract used for the analysis. Chromatograms were developed in the solvent system petroleum ether (b.p. 45 to 66 °C)–diethyl ether–glacial acetic acid (85:15:1). Similar separations were obtained in the solvent systems *n*-hexane–diethyl ether–glacial acetic acid (70:30:2) and petroleum ether (b.p. 40 to 60 °C)–diethyl ether–glacial acetic acid (90:10:1). If lower b.p. petroleum ether is used and when the LCN-A spot is difficult to interpret, it is advisable to dry the plates and re-run them in pure methanol. In methanol most of the lipids, but not lipid LCN-A, moved with the solvent front. After the chromatograms had been developed the plates were dried for 3 to 4 h at 35 to 45 °C and then exposed to iodine vapour. The lipid spots appeared after about 5 min. All analyses were made in duplicate or in triplicate.

Arabinose and diaminopimelic acid. Acid hydrolysates of the mycelium were examined for arabinose and diaminopimelic acid by paper chromatography by means of the techniques

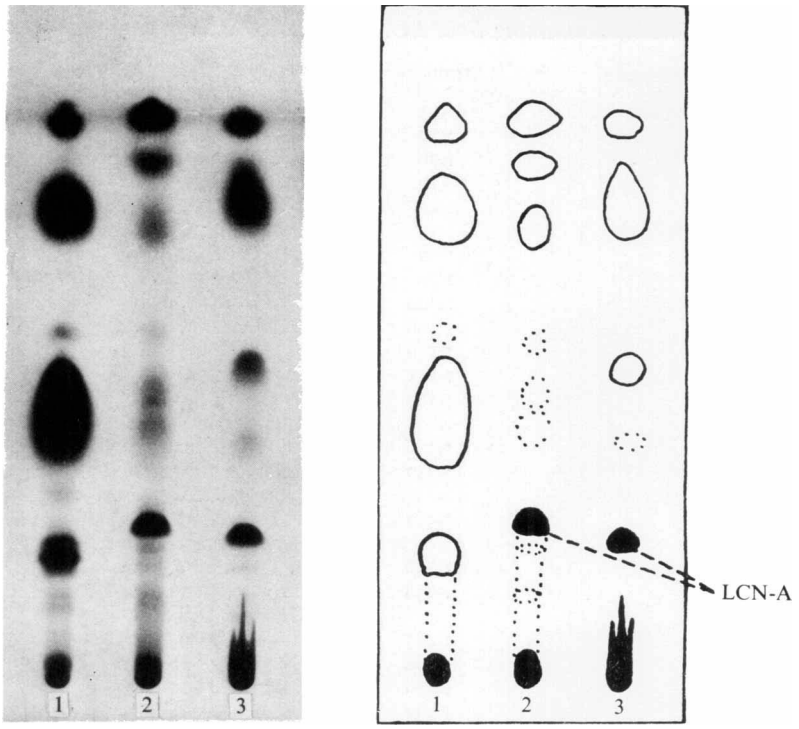

Fig. 1. Thin-layer chromatogram of cell lipids of the standard strains: 1, *Streptomyces griseus* 22; 2, *Nocardia asteroides* USA; 3, *N. calcarea* IMET 7018. Solvent system: petroleum ether–diethyl ether–glacial acetic acid (85:15:1).

described by Murray & Proctor (1965) and Becker, Lechevalier, Gordon & Lechevalier (1964). Undefatted cells as well as those remaining after lipid extraction were used in these experiments.

RESULTS

The cells of the 143 cultures listed in Table 1 contained lipid LCN-A, arabinose and *meso*-diaminopimelic acid. Some of the strains gave an LCN-A spot which had a slightly lower R_F value than that of the standard *Nocardia asteroides* USA strain. The position of this analogue in chromatograms corresponded with the LCN-A spot of the standard *N. calcarea* IMET 7017 (Fig. 1). This analogue, lipid LCN-A 'calcarea' was found in *N. calcarea* and in some of the strains of the '*Mycobacterium*' *rhodochrous* complex. The remaining strains contained the normal lipid LCN-A 'asteroides' type.

The 55 cultures which did not contain lipid LCN-A are shown in Table 2. None of these cultures can be accommodated in the genus *Nocardia sensu novo* but belong either to the new genera *Actinomadura* and *Oerskovia* or to the established taxa *Mycobacterium* and *Streptomyces*. The other chemical data indicated that the streptomycetes had a wall Type I, and the actinomaduras a wall Type III (Lechevalier & Lechevalier, 1970*a*).

Twenty strains received as *Nocardia* spp. did not contain lipid LCN-A. Six of these organisms had a wall Type I, ten a wall Type III, and the remaining four possessed a wall Type IV. Identical results were obtained with defatted and undefatted cells.

Table 1. *Strains containing lipid LCN-A, arabinose and meso-diaminopimelic acid*

Name of strain	Strain no.	Source
Nocardia asteroides	N13	NCTC 8595
	N70	IAM 0374
	N76	S. T. Williams, Liverpool University, E13
	N77	S. T. Williams, E15
	N96	R. E. Gordon, Rutgers University, New Brunswick, U.S.A., W3300
	N97	R. E. Gordon, N659
	N98	R. E. Gordon, 618
	N100	R. E. Gordon, 652
	N106	R. E. Gordon, A9504
	N119	CBS 255. 58
	N121	CBS 333. 51
	N127	CBS 248. 33
	N204	ATCC 7372
	N216	J. E. Thiemann, Lepetit, Milan, S547
	N317	ATCC 19247. Suggested working type (Sneath & Skerman, 1966)
	N364	M. Tsukamura, The National Sanitorium, Aichi-ken 474, Japan, R399
	N366	M. Tsukamura, ATCC 9970
	N458	L. Ajello, Center for Disease Control, Atlanta, Georgia, U.S.A., 45-765-70
	N483	L. Ajello, 45-995-70
	N486	L. Ajello, 45-1005-70
	N489	L. Ajello, 45-1007-70
	N492	L. Ajello, 45-1065-70
	N509	L. Ajello, 45-1109-70
	N512	L. Ajello, 45-54-71
	N518	L. Ajello, 45-231-71
	N519	L. Ajello, 45-246-71
	N520	L. Ajello, 45-274-71
	N528	L. Ajello, 45-379-71
N. brasiliensis	N48	R. E. Gordon, 744
	N118	CBS 438. 64
	N318	ATCC 19296, uncertain cotype (Sneath & Skerman, 1966)
	N367	M. Tsukamura, R432
	N368	M. Tsukamura, R887
	N425	R. E. Gordon, 605
	N426	R. E. Gordon, 774A
	N427	R. E. Gordon, 774B
	N428	R. E. Gordon, 1336
	N429	R. E. Gordon, 731
	N438	R. E. Gordon, 1108
	N439	R. E. Gordon, 3488
	N464	Institut Pasteur, Paris, 700
	N465	IP 701
	N466	IP 704
	N467	IP 708
	N468	IP 723
	N469	IP 748
	N470	A. Gonzáles Ochoa, Instituto de Salubridad, Mexico 17, 4060
	N471	A. Gonzáles Ochoa, 4115
	N474	A. Gonzáles Ochoa, 4212
	N475	A. Gonzáles Ochoa, 4023
	N476	A. Gonzáles Ochoa, 4025
	N481	J. A. Serrano, Universidad De Los Andes, Merida, 1548

Table 1 (*cont.*)

Name of strain	Strain no.	Source
N. brasiliensis	N482	L. Ajello, 45-944-70
	N488	L. Ajello, 45-1012-70
N. calcarea	N41	NCIB 8863
N. caviae	N21	CCM 197
	N36	NCTC 1934, cotype (Sneath & Skerman, 1966)
	N231	R. Olds, Cambridge University, CN749
	N232	R. Olds, CN751
	N313	IP 751
	N314	IP 771
	N369	M. Tsukamura, R1315
	N370	M. Tsukamura, R1316
	N371	M. Tsukamura, R416
	N430	R. E. Gordon, 1370
	N431	R. E. Gordon, 737
	N432	R. E. Gordon, 416
	N440	R. E. Gordon, 1355
	N441	R. E. Gordon, 1316
	N442	R. E. Gordon, 424
	N459	IP 318
	N460	IP 772
N. congolensis	N15	NCTC 5175
N. convoluta	N95	A. Gonzáles Ochoa, 98
	N140	J. Antheunisse, Wageningen, Holland, ATCC 4275
N. cuniculi	N16	NCTC 1935
N. farcinica	N358	M. Tsukamura, M133
	N359	M. Tsukamura, M205
	N360	M. Tsukamura, M175
N. gardneri	N29	IFO 3385
N. marina	N145	M. Turner, Nottingham University, 36
N. narashinoensis	N69	IAM 0113
N. pasteuroides	N94	A. Gonzáles Ochoa, 35
N. pelletieri	N78	S. T. Williams, E21
N. petroleophila	N43	NCIB 9438
N. pretoriana	N237	I. Uesaka, Kyoto University, Japan, 194
N. rhodnii	N219	C. da Silva Lacaz, São Paulo University, Brazil
	N220	C. da Silva Lacaz
	N443	P. Hill, Edinburgh University, A/1
	N444	P. Hill, A/0
	N446	P. Hill, B/1
N. sylvodorifera	N217	J. E. Thiemann, S546
N. uniformis	N3	NCIB 9631
N. vaccinii	N33	NCPPB 954, Holotype (Sneath & Skerman, 1966)
Nocardia sp.	N91	H. Veldkamp, Groningen University, A86
	N350	T. Watson, Liverpool University, A32
	N403	H. Weyland, Institut für Meeresforschung, Bremerhaven, 31
	N478	L. Ajello, 45-908-70
	N521	CBS C568
	N522	CBS 5085

Table 1 (*cont.*)

Name of strain	Strain no.	Source
Mycobacterium rhodochrous	N4	NCIB 9664
	N5	NCIB 9701
	N7	NCIB 10027
	N22	CCM 3245
	N25	CCM 279
	N26	CCM 198
	N27	CCM 278
	N28	CCM 269
	N30	R. E. Gordon, A12974
	N31	R. E. Gordon, W21
	N55A	R. E. Gordon, 817
	N55B	Mucoid variant of 55A
	N56	R. E. Gordon, 1256
	N57	R. E. Gordon, 1293S
	N58A	R. E. Gordon, 1257
	N58B	Dry variant of 58A
	N59	R. E. Gordon, 1240
	N60	R. E. Gordon, 1293R
	N61	R. E. Gordon, W3408
	N62	R. E. Gordon, 768
	N63	R. E. Gordon, 463
	N65	R. E. Gordon, A7698
	N66	NCTC 8139
	N67	NCTC 10210
	N73	S. T. Williams, E40
	N75	LA 1609
	N108	R. E. Gordon, A4277
	N109	R. E. Gordon, 494
	N110	R. E. Gordon, W3639
	N112	V. B. D. Skerman, Queensland University, Australia, 121
	N113	V. B. D. Skerman, 134
	N123	CBS 334. 51
	N146	M. Turner, 39
	N240	NCTC 8571
	N324	ATCC 15998
	N325	G. Castelnuova, Instituto Superiore Di Sanita, Rome, 906B
	N326	G. Castelnuova, 107
	N420	A. Tacquet, Institut Pasteur, Lille, 906
	N422	A. Tacquet, 107
	N424	J. Norris, Millstead Laboratory, Sittingbourne, Kent, 330
	N447	R. Bönicke, Forschungs'nstitut Borstel, SN5108
	N450	R. Bönicke, SN5303
	N451	R. Bönicke, SN5302

ATCC = American Type Culture Collection, Rockville, Maryland, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Baarn, Netherlands; IAM = Institute of Applied Microbiology, University of Tokyo, Japan; IFO = Institute of Fermentation, Osaka, Japan; LA = Institut d'Hygiene, Lausanne, Switzerland; NCIB = National Collection of Industrial Bacteria, Aberdeen; NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire; NCTC = National Collection of Type Cultures, London.

Table 2. *Strains which do not contain lipid LCN-A but which may have arabinose and/or diaminopimelic acid*

Name of strain	Strain no.	Source	Arabinose	DAP
Actinomadura dassonvillei	N238	NCTC 10489	−	+(DL, LL)
	N287	NCTC 10488	−	+(DL)
	N433	R. E. Gordon, 714	−	+(DL)
	N435	R. E. Gordon, 1322	−	+(DL)
	N436	R. E. Gordon, 1289	−	+(DL)
	N437	R. E. Gordon, 575	−	+(DL)
A. madurae	N17	NCTC 1070	−	+(DL)
	N80	S. T. Williams, E23	−	+(DL, LL)
	N81	S. T. Williams, E24	−	+(DL)
	N374	M. Tsukamura, Sal. 1	−	+(DL)
A. pelletieri	N18	NCTC 10000	−	+(DL, LL)
	N49	R. E. Gordon, 513	−	+(DL, LL)
	N79	S. T. Williams, E22	−	+(DL)
	N282	I. G. Murray, London School Tropical Medicine, 1067	−	+(DL)
	N298	IP 726	−	+(DL)
	N461	IP 390	−	+(DL)
	N462	IP 389	−	+(DL)
	N463	IP 388	−	+(DL)
Mycobacterium abscessus	M29	LA 948	+	+(DL)
M. chitae	M26	NCTC 10495, paratype	+	+(DL)
M. fortuitum	N294	ATCC 6841	+	+(DL)
M. giae	M38	LA 82	+	+(DL)
M. phlei	N290	NCTC 8151	+	+(DL)
M. salmoniphilum	M32	LA 1263, type	+	+(DL)
Nocardia aerocolonigenes	N538	NRRL B3298, type	±	+(DL)
N. alba shoen	N235	I. Uesaka, 113	−	+(LL)
N. apis	N8	NCIB 9378	−	+(LL)
N. capreola	N540	NRRL 2773, type	±	+(DL)
N. cellulans	N40	NCIB 8868	−	+(DL)
N. coeliaca	N1	NCIB 9574	+	+(DL)
N. farcinica	N34	NCTC 4524, cotype (Sneath & Skerman, 1966)	+	+(DL)
	N120	CBS 223. 60	+	+(DL)
N. formica	N203	ATCC 14811	−	+(LL)
N. gibsonii	N205	ATCC 6852	−	+(LL)
N. italica	N9	NCIB 9386	−	+(DL, LL)
	N531	CBS 609. 67	−	+(DL)
N. lurida	N2	NCIB 9601	−	+(DL)
N. orientalis	N539	NRRL 2540, type	±	+(DL)
N. piedadensis	N207	ATCC 15747	−	+(LL trace)
N. polychromogenes	N72	LA 1610	−	+(DL)
N. rangoonensis	N206	ATCC 6860	−	+(LL)
N. rugosa	N44	NCIB 8926	+	+(DL)
N. saturnea	N45	NCIB 9437	−	+(DL, LL)
N. tenuis	N117	CBS 260. 35	+	+(DL)
Oerskovia turbata	N50	D. M. Webley, Macauley Institute, Aberdeen, strain C	−	−
	N51	D. M. Webley, strain B	−	−

Table 2 (*cont.*)

Name of strain	Strain no.	Source	Arabinose	DAP
Oerskovia turbata	N414	M. P. Lechevalier, Rutgers University, 891	–	–
	N415	M. P. Lechevalier, 689	–	–
	N416	M. P. Lechevalier, 17-11	–	–
	N418	M. P. Lechevalier, 713-3	–	–
	N419	M. P. Lechevalier, 713-4	–	–
Streptomyces griseus	N87	S. T. Williams, A24	–	+(LL)
S. netropsis	N227	S. T. Williams, A21	–	+(LL)
S. somaliensis	N20	NCIB 3236	–	+(LL)
S. viridochromogenes	N226	S. T. Williams, A29	–	+(LL)

NRRL = Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

DISCUSSION

All the strains found, on phenetic evidence, to belong to the genus *Nocardia* (Goodfellow, 1971) contained lipid LCN-A, arabinose and *meso*-diaminopimelic acid. Actinomadura and oerskovia strains did not possess lipid LCN-A or arabinose though cells of the former contained *meso*-diaminopimelic acid and a few a trace of arabinose. Certain actinomadura strains showed traces of L-diaminopimelic acid which suggested a possible link between the genera *Actinomadura* and *Streptomyces*. The chemotaxonomic characters, particularly the method to detect lipid LCN-A, allow nocardias to be reliably differentiated from actinomadura and oerskovia strains.

It is common knowledge that it is sometimes difficult to distinguish between nocardia and streptomyces strains. Streptomycetes which have lost the ability to produce aerial hyphae are not easy to separate from nocardias; similarly, freely sporulating strains of *Nocardia asteroides* cannot readily be differentiated, on morphological grounds, from streptomycetes. Strains can now be referred to one or other of these genera depending upon whether or not they contain lipid LCN-A and the isomer of diaminopimelic acid that they possess.

Another difficult borderline is that between the genera *Nocardia* and *Mycobacterium*. All the strains in these taxa have a wall Type IV in common, and fast growing mycobacteria cannot always be separated from nocardias by morphological and biochemical criteria. Mycobacteria, however, contain mycolic acids *sensu stricto* and nocardias nocardomycolic acids (Asselineau, 1966). The detection of differences in the structure of these mycolic acids involves the use of techniques such as mass spectrometry (Etémadi, 1967*b*) and pyrolysis gas chromatography (Lechevalier *et al.* 1971). These techniques are not difficult, but do require the use of equipment still lamentably beyond the resources of many diagnostic laboratories. The fact that mycobacteria do not contain lipid LCN-A is, therefore, especially relevant because the method to detect this lipid provides a simple and useful diagnostic test for differentiating nocardias from mycobacteria.

'*Mycobacterium*' *rhodochrous* has for a long time been a taxonomic enigma and even after the thorough study by Gordon (1966) its genetic location is open to question. The preliminary work of Lanéelle *et al.* (1965) was confirmed when Lechevalier *et al.* (1971) detected nocardomycolic acids in '*M.*' *rhodochrous* strains and on this evidence temporarily endorsed their inclusion in the genus *Nocardia*. The detection of lipid LCN-A in '*M.*' *rhodochrous* strains is further evidence of a close taxonomic affinity with nocardias. Numerical taxonomic data, however, have shown that strains of '*M.*' *rhodochrous* form a taxon which is quite distinct from clusters containing nocardias (Goodfellow, 1971) but even more sharply

separated from a cluster containing rapidly growing mycobacteria (Goodfellow, Fleming & Sackin, 1971). It might, therefore, be important that in certain '*M.*' *rhodochrous* strains the LCN-A spot had a slightly lower R_F value than that of the reference lipid LCN-A 'asteroides' type. A further analogue with an even lower R_F value has been found in strains of corynebacteria (Mordarska & Mordarski, 1970). Further work, on additional corynebacteria and '*M.*' *rhodochrous* strains, is needed to try to differentiate between the analogues and lipid LCN-A. The best differentiation, to date, has been obtained by Mordarska & Mordarski (1970) by threefold chromatography in the solvent system *n*-hexane–diethyl ether–glacial acetic acid (70:30:1). The results of the lipid and phenetic studies make us reluctant, at present, to assign strains in the '*M.*' *rhodochrous* complex to the genus *Nocardia*.

An understanding of the chemical nature of the LCN-A spots should offer new leads for the separation of lipid LCN-A and its analogues. Although preliminary data (Mordarska & Réthy, 1970) have shown that the LCN-A spot is heterogeneous, the main components have not yet been analysed critically. We are, therefore, not yet able to say how nearly the LCN-A spots resemble the mycolic acids found by Lechevalier *et al.* (1971).

Twenty strains received bearing the epithet *Nocardia* did not contain lipid LCN-A and gave different responses in the other chemical analyses. On the basis of these chemotaxonomic data most of these strains can be provisionally assigned to the genera *Actinomadura* and *Streptomyces*. Some of these strains may represent transitional forms between established taxa. *Nocardia rangoonensis* and *N. piedadensis* were considered to be streptomycetes, but in contrast to typical streptomycetes, though like nocardias, they are unable to hydrolyse starch (Modarska, Wieczorek & Jaworska, 1970). *Nocardia farcinica* strain N34, NCTC 4524, did not contain lipid LCN-A and should not be retained in the genus *Nocardia*. This strain is allegedly a duplicate of the type strain ATCC 3318. Our results, therefore, lend support to the proposals of Lechevalier *et al.* (1971) that *N. farcinica* be considered a *nomen dubium*.

The nocardioform bacteria remain a formidable group. It is likely that the discovery of additional chemotaxonomic characters, particularly involving lipids, will have an important role to play in the taxonomy of these organisms.

One of us, M. G., thanks The Wellcome Trust for a Travel Grant which supported a trip to Wrocław. This work was also supported by Medical Research Council Grant G 970/112/B. Finally, the help given, so willingly, by Grace Hedley and Barbara Lanteck has not been forgotten.

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