Numerical and Chemical Classification of *Nocardia amarae*

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Twenty-one strains of *Nocardia amarae* and marker cultures of *Mycobacterium*, *Nocardia*, *Rhodococcus* and the 'aurantiaca' taxon were subjected to numerical phenetic analyses using 92 unit characters. The data were examined using the simple matching (S_{SM}), Jaccard (S_J) and pattern (D_p) coefficients and clustering was achieved using the unweighted average linkage algorithm. Neither cluster nor aggregate cluster composition was markedly affected by the coefficient used or by test error, estimated at 1.5%. The *N. amarae* strains formed a distinct and homogeneous cluster which showed its highest similarity to phenotypes equated with *Nocardia asteroides*, *Nocardia brasiliensis* and *Nocardia otitidis-caviarum*. The non-hydroxylated fatty acid composition and overall size of the mycolic acids was similar to that found to be characteristic of *Nocardia sensu stricto*, though the long-chain in the 2-position of the mycolic acids was relatively much richer in monounsaturated components. *Nocardia amarae*, in containing dihydrogenated menaquinones with nine isoprene units, is clearly distinguished from established representatives of *Nocardia*.

INTRODUCTION

Nocardia amarae was proposed by Lechevalier & Lechevalier (1974) for novel actinomycetes isolated in large numbers from foam formed on the surface of aeration tanks in activated-sludge sewage-treatment plants. The organism contained meso-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), mycolic acids with characteristic unsaturated branches in the 2-position and was considered to be related to *Nocardia* ('*Mycobacterium*') *rhodochrous* on the basis of chemical and physiological criteria. The genus *Rhodococcus* was subsequently resurrected for strains labelled 'M'. *rhodochrous* (Tsukamura, 1974; Goodfellow & Alderson, 1977).

In the absence of extensive systematic studies little is known of the detailed relationships of *N. amarae* and other wall chemotype IV, mycolic acid-containing strains classified in the genera *Corynebacterium sensu stricto*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and the 'aurantiaca' taxon (Goodfellow & Minnikin, 1977, 1981; Goodfellow *et al.*, 1978; Minnikin *et al.*, 1978; Minnikin & Goodfellow, 1980, 1981). *Nocardia amarae* strains do, however, contain fatty acids and polar lipids similar to those of *Nocardia* and *Rhodococcus* (Lechevalier *et al.*, 1977), have a DNA base composition within the range 60 to 66 mol % guanine plus cytosine, and with one exception form a distinct DNA homology group (Mordarski *et al.*, 1977; Goodfellow & Minnikin, 1981). Further, in preliminary DNA:rRNA pairing experiments the type strain of *N. amarae* was more closely related to *Nocardia* strains than to reference cultures of *Mycobacterium* and *Rhodococcus* (Mordarski

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Table 1. *Designation and source of strains assigned to subcluster 3A and cluster 5 in the S_{SM}/UPGMA analysis*

(a) Cultures assigned to cluster 3A (<i>Nocardia amarae</i> ; all from abnormal foam)	
*N667	<i>Nocardia amarae</i> ; M. P. Lechevalier (M.P.L.), Rutgers University, New Brunswick, U.S.A., Se6 (ATCC 27808, IMRU W3960); Andover (Miami), Fla., U.S.A.
N667A	<i>N. amarae</i> ; duplicate of strain N667
N774	<i>N. amarae</i> ; M.P.L., Se53; Riverdale (Miami), Fla., U.S.A.
N775	<i>N. amarae</i> ; M.P.L., Se64; Ocean Township, N.J., U.S.A.
N776	<i>N. amarae</i> ; M.P.L., Se91; Austin, Tex., U.S.A.
N777	<i>N. amarae</i> ; M.P.L., Se97; Bordentown Township, N.J., U.S.A.
N778	<i>N. amarae</i> ; M.P.L., Se107; Eversham, N.J., U.S.A.
N779	<i>N. amarae</i> ; M.P.L., Se110; Delran, N.J., U.S.A.
N780	<i>N. amarae</i> ; M.P.L., Se117; Matawan, N.J., U.S.A.
N781	<i>N. amarae</i> ; M.P.L., Se149B; Jamaica Bay, N.J., U.S.A.
N782	<i>N. amarae</i> ; M.P.L., Se160; Arlington, Va., U.S.A.
N782A	<i>N. amarae</i> ; duplicate of strain N782
N783, N784	<i>N. amarae</i> ; M.P.L., Se230, Se238; Chatham-Madison, N.J., U.S.A.
N785	<i>N. amarae</i> ; M.P.L., Se248; Enfield, Conn., U.S.A.
N786	<i>N. amarae</i> ; M.P.L., Se279; Middletown, N.J., U.S.A.
N787	<i>N. amarae</i> ; M.P.L., Se291; Somerset-Raritan, N.J., U.S.A.
N788	<i>N. amarae</i> ; M.P.L., Se300; Union Beach, N.J., U.S.A.
N789	<i>N. amarae</i> ; M.P.L., Se306; Sewickley, Pa., U.S.A.
N790	<i>N. amarae</i> ; M.P.L., Se325; Bernardsville, N.J., U.S.A.
N791	<i>N. amarae</i> ; M.P.L., 100.60; Rodeo, Calif., U.S.A.
(b) Cultures assigned to cluster 5A (<i>Corynebacterium diphtheriae</i>)	
*C67	<i>Corynebacterium diphtheriae</i> var. <i>minimus</i> ; NCTC 10681
C14	<i>C. diphtheriae</i> var. <i>intermedius</i> ; NCTC 3987
(c) Cultures assigned to cluster 5B (<i>Corynebacterium bovis</i>)	
*C12	<i>Corynebacterium bovis</i> ; NCTC 3224
C103	<i>C. bovis</i> ; J. E. Shreeve, Central Veterinary Laboratory, Weybridge, U.K., 120B
(d) Cultures assigned to cluster 5C (<i>Corynebacterium pseudotuberculosis</i>)	
C145	<i>Corynebacterium pseudotuberculosis</i> ; H. R. Carne, Pathology Department, Cambridge University, U.K., OV 137c
C146	<i>C. pseudotuberculosis</i> ; H. R. Carne, OV 137d
C150	<i>C. pseudotuberculosis</i> ; H. R. Carne, OV 1134
C152	<i>C. pseudotuberculosis</i> ; H. R. Carne, Cap 16
(e) Single member cluster	
C19	<i>Corynebacterium pseudodiphtheriticum</i> ; NCTC 231

* Type strain.

et al., 1980). However, some strains of *N. amarae* have been reported to lack the galactose characteristic of *Nocardia sensu stricto* (Gordon *et al.*, 1978), while the type strain was not lysed by nocardiphages (Williams *et al.*, 1980) and was only loosely associated with true nocardiae in numerical phenetic analyses (Orchard *et al.*, 1977; Orchard & Goodfellow, 1980; Goodfellow & Pirouz, 1982).

In the present study representatives of *N. amarae* and marker cultures of *Corynebacterium sensu stricto*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and the 'aurantiaca' taxon were compared using numerical phenetic techniques. The fatty acid, mycolic acid and isoprenoid quinone composition of the *N. amarae* strains was also determined.

METHODS

Strains, and collection and coding of data. Twenty-one *N. amarae* strains, 9 corynebacteria, 10 rapidly growing mycobacteria, 13 nocardiae, 62 rhodococci and 8 aurantiaca strains were studied. Only histories of the *Corynebacterium* and *N. amarae* strains are given (Table 1) as those of the other test organisms have already been

cited (Goodfellow *et al.*, 1978). All of the cultures were maintained on glucose yeast extract agar (Gordon & Mihm, 1962) at room temperature.

The corynebacteria and *N. amarae* strains were examined for 92 unit characters using tests described elsewhere (Goodfellow, 1971; Lacey & Goodfellow, 1975; Goodfellow & Alderson, 1977), and the results were added to the data bank existing on the other organisms (Goodfellow *et al.*, 1978).

Computer analysis. Data were analysed using the Clustan 1A program (Wishart, 1968) on an IBM 370/180 computer, using the simple matching coefficient (S_{SM} ; Sokal & Michener, 1958), which includes both positive and negative matches, the Jaccard coefficient (S_J ; Sneath, 1957), which includes positive matches only, and the pattern coefficient (D_p ; Sneath, 1968), which seeks to allow for differences in growth rates, incubation periods and similar factors which might contribute to overall dissimilarity between strains. Data were sorted using the unweighted average linkage (UPGMA) algorithm (Sneath & Sokal, 1973); those from the S_{SM} /UPGMA analysis are presented as an abbreviated dendrogram (Fig. 1).

Lipid analyses. The *N. amarae* strains were grown in shake culture at 30 °C for up to 7 d in modified Sauton's medium (Mordarska *et al.*, 1972), checked for purity at maximum growth, killed by shaking overnight with formalin (1%, v/v), separated by centrifugation, washed with distilled water, and freeze-dried.

Freeze-dried bacteria (50 mg) were degraded by acid methanolysis, and hexane extracts were examined for long-chain components by single-dimensional thin-layer chromatography (t.l.c.), as described previously (Minnikin *et al.*, 1975), using Merck silica gel H (0.5 mm layers) and a developing mixture of petroleum ether (b.p. 60–80 °C)/diethyl ether (85:15, v/v). Two-dimensional t.l.c. of methanolysates was performed using 10 × 10 cm plates cut from silica gel 60 F₂₅₄ aluminium sheets (Merck no. 5554) and a triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction, followed, in the second direction, by a single development with toluene/acetone (97:3, v/v) (Minnikin *et al.*, 1980).

Long-chain components were isolated by preparative t.l.c. on layers (1 mm) of Merck silica gel PF₂₅₄ + 366, separated bands being detected with ultraviolet light (366 nm). Long-chain fatty acid methyl esters were analysed by isothermal gas chromatography on a Perkin-Elmer F11 flame-ionization instrument using nitrogen as carrier gas and stainless steel columns (0.125 in, o.d.) packed with 10% Silar 10C on 100–120 mesh Gas-Chrom Q (6 m long; Applied Science Laboratories) at 210 °C or 2.5% OV-1 on 80–100 mesh Chromosorb G AW-DMCS (2 m long; Phase Separations) at 180 °C. Analyses using these polar and non-polar columns allowed the positive identification of the major long-chain fatty acid methyl esters from *N. amarae*, but for the quantification of minor amounts of methyl esters having exceptionally long chains a shorter (1 m) OV-1 column was used, with a programmed temperature increase at 6 °C min⁻¹ from 120 to 300 °C.

Mycolic acid methyl esters were examined by pyrolysis gas chromatography using the 6 m Silar 10C and 2 m OV-1 columns, and by mass spectrometry on an AEI MS9 instrument with a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 190 to 220 °C. The solubility of mycolic acids of *N. amarae* N780 in ethanol/diethyl ether (2:1, v/v) was assessed by the method of Hecht & Causey (1976).

Isoprenoid quinones were extracted, purified and analysed by mass spectrometry as described by Collins *et al.* (1977).

RESULTS

Clustering of strains using the S_{SM} , S_J and D_p coefficients and the UPGMA algorithm

The corynebacteria, mycobacteria, nocardiae, rhodococci and aurantiaca strains were recovered in five aggregate clusters defined at or just below the 70% similarity level in the S_{SM} /UPGMA analysis (Fig. 1). Both aggregate clusters and subclusters were named, where possible, after the type or authentic strains they contained (Table 1); the codes for the aggregate clusters are the same as those used in other numerical phenetic surveys (Goodfellow & Alderson, 1977; Goodfellow *et al.*, 1978; Goodfellow *et al.*, 1982). The composition of the *Mycobacterium*, *Rhodococcus* and 'aurantiaca' aggregate clusters were as in previous analyses (Goodfellow *et al.*, 1978, 1982) and will not be considered here.

The *N. amarae* strains formed a distinct homogeneous cluster lying on the periphery of the *Nocardia* aggregate cluster but separate from phena corresponding to *Corynebacterium sensu stricto*, *Mycobacterium*, *Rhodococcus* and the 'aurantiaca' taxon (Fig. 1). The corynebacteria formed the most diffuse aggregate cluster containing three clusters – 5A, 5B and 5C – corresponding to *C. diphtheriae*, *C. bovis* and *C. pseudotuberculosis*, respectively (Table 1).

Essentially similar results were obtained in both the S_J /UPGMA and D_p /UPGMA analyses, although in the latter the *N. amarae* strains fell outside the aggregate *Nocardia* cluster and the *C. bovis* and *C. diphtheriae* clusters were recovered as a single phenon.

Fig. 1. A simplified dendrogram showing the relationships between aggregate clusters and subclusters based on the S_{SM} coefficient and the average linkage algorithm (UPGMA).

Reproducibility of results

The inclusion of two duplicate cultures of *N. amarae* (Table 1) in the analysis enabled experimental test error to be estimated. The probability (p) of an erroneous result averaged 1.5%, equal to an observed S_{SM} value of about 97% between duplicate strains.

Differentiation of *Nocardia amarae*, *N. asteroides*, *N. brasiliensis* and *N. otitidis-caviarum*

The percentage of positive responses to each test for the *N. amarae* strains is compared in Table 2 with data obtained in corresponding studies on *N. asteroides*, *N. brasiliensis* and *N. otitidis-caviarum* (Goodfellow, 1971; Lacey & Goodfellow, 1975). The 15 tests with the greatest resolving power for the differentiation of the four species are given in Table 3. It is evident from Table 2 that *N. asteroides* is heterogeneous, a fact that was underlined in a recent numerical phenetic survey (Orchard & Goodfellow, 1980).

Lipid analyses

Whole-organism acid methanolysates from all the *N. amarae* strains gave similar patterns on single- and two-dimensional t.l.c.; an example of the latter is given in Fig. 2 for the type strain N667. The principal long-chain components in *N. amarae* corresponded to non-hydroxylated fatty acids and mycolic acid methyl esters (Minnikin *et al.*, 1980) but

minor amounts of other components were detected in most strains (Fig. 2). These minor long-chain compounds were not characterized, but the least polar components (Fig. 2) were possibly nocardols – long-chain alcohols closely related to mycolic acids (Minnikin *et al.*, 1980). The non-hydroxylated fatty acid methyl esters were isolated by preparative t.l.c. and the results of gas chromatographic analyses on both polar and non-polar phases are shown in Table 4. The principal non-hydroxylated fatty acids in *N. amarae* were C₁₆ and C₁₈ straight-chain saturated and monounsaturated acids and an acid whose ester co-chromatographed with authentic 10-methyloctadecanoate prepared from *Mycobacterium avium*. Minor amounts of methyl esters having chains longer than 18 carbons were not positively identified (Table 4).

Mycolic acid methyl esters from *N. amarae* strains were also isolated from methanolysates by preparative t.l.c. and subjected to mass spectrometry and pyrolysis gas chromatography. Methyl esters of mycolic acids having substantial amounts of unsaturated chains in the 2-position fragment on mass spectrometry as shown in Fig. 3 (Goodfellow *et al.*, 1978; Collins *et al.*, 1982). The highest peaks in the mass spectra correspond to anhydromycolates resulting from the elimination of a molecule of water from the parent mycolate. Cleavage according to *a* and *a'* (Fig. 3) involves net transfer of the hydroxyl hydrogen atom to produce long-chain methyl esters and aldehydes (meroaldehydes). The elements of methanol are readily lost from monounsaturated esters formed by cleavage *a* to give characteristic fragments X (Fig. 3) (Goodfellow *et al.*, 1978; Collins *et al.*, 1982). The overall sizes of the side-chains R₁ (Fig. 3) are also revealed by the presence of fragments Y due to cleavage *b*.

The characteristic mass spectral fragmentation pattern, outlined above, is exemplified by the partial mass spectrum of the methyl mycolates from *N. amarae* N667 shown in Fig. 4. Partial mass spectra of the mycolic esters from the remaining strains of *N. amarae* (Table 1) have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, as Supplementary Publication No. SUP 28012 (5 pages). (Copies may be obtained from the BLLD on demand; where possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.) Peaks corresponding to anhydromycolates (Fig. 3) in the mass spectra of the mycolic acid methyl esters from all representatives of *N. amarae*, with the exception of strain N786, were closely similar to those shown in Fig. 4, and a summary of their distribution is given in Table 5. The anhydromycolate fragments in the mass spectrum of the mycolic esters from *N. amarae* N786 were significantly different (Table 6). Peaks corresponding to pyrolytic release of meroaldehydes and long-chain esters (Etémadi, 1967; Figs 3, 4) were observable in the mass spectra of all the mycolic esters analysed. The competing pathways (Fig. 3) leading to peaks derived from long-chain esters hamper the quantitative estimation of the composition of the long-chains in the 2-position of the mycolic acids (Collins *et al.*, 1982) and the precise composition was therefore established by pyrolysis gas chromatography (Etémadi, 1967); the results are shown in Table 7. The mycolic acids of *N. amarae* N780 were soluble in ethanol/diethyl ether (2:1, v/v) as tested by the procedure of Hecht & Causey (1976).

Extracts of the *N. amarae* strains contained menaquinones which co-chromatographed on t.l.c. with vitamin K. The most significant peak in the spectra occurred at *m/e* 786 corresponding to a dihydrogenated menaquinone with nine isoprene units, abbreviated as MK-9(H₂); smaller peaks at *m/e* 650, 716, 718 and 784 correspond to the minor components MK-7(H₂), MK-8, MK-8(H₂) and MK-9 (Table 8).

DISCUSSION

In the numerical analysis, phenon composition was not markedly affected either by the statistics used or by the 1.5% test error. In the *S*_{SM}, *S*_J and *D*_P analyses with UPGMA, the *N. amarae* strains were recovered as a homogeneous cluster which was clearly separated from the phena in aggregate clusters corresponding to *Corynebacterium sensu stricto*,

Table 2. *A comparison of the characteristics of Nocardia amarae with those of well established species of Nocardia*

Results show the percentage frequencies of positive characters.

Character	<i>N. amarae</i> (19 strains)	<i>N. asteroides</i> * (42 strains)	<i>N. brasiliensis</i> * (10 strains)	<i>N. otitidis-caviarum</i> * (10 strains)
Staining and colony morphology:				
Strongly acid-fast	0	46	100	50
Aerial hyphae, sparse	90	98	100	90
Aerial hyphae, moderate	74	77	100	60
Aerial hyphae, abundant	37	62	100	20
Colonies white, cream, tan	100	17	0	100
Colonies pink, red, orange	0	77	70	0
Diffusible exopigment	0	43	100	90
Elevation convex	27	12	0	20
Elevation irregular	85	89	100	80
Margin entire	5	0	0	0
Margin filamentous	95	100	100	100
Degradation of:				
Casein	16	0	100	0
Elastin	0	0	100	0
Hypoxanthine	0	5	100	90
Tween 20	100	100	20	90
Tween 40	100	97	10	70
Tween 60	100	84	10	70
Tyrosine	0	0	100	0
Xanthine	0	0	0	100
Growth on sole carbon source (at 1 g l ⁻¹):				
L-Arabinose	0	0	50	0
Cellobiose	0	0	0	30
Ethanol	100	48	100	0
Fructose	90	91	80	100
Galactose	16	24	90	0
Glycerol	100	82	90	10
Inositol	85	2	100	90
Inulin	11	0	0	0
Lactose	0	0	10	0
Maltose	95	74	100	60
Mannose	100	86	100	40
Mannitol	90	53	90	100
Rhamnose	90	36	0	0
Salicin	100	10	30	20
Sorbitol	0	7	0	10
Sucrose	95	7	70	20
Trehalose	100	26	100	40
Xylose	0	0	30	0
Growth on sole carbon source (at 0.1 g l ⁻¹):				
Acetamide	0	7	0	10
<i>p</i> -Cresol	0	2	0	10
<i>m</i> -Hydroxybenzoic acid	0	14	0	0
<i>p</i> -Hydroxybenzoic acid	58	0	0	0
Pimelic acid	100	0	10	0
Sebacic acid	100	84	90	100
Testosterone	0	82	0	100
L-Tyrosine	0	0	70	0
Sodium adipate	90	53	0	0
Sodium benzoate	0	12	0	0
Sodium citrate	0	12	90	50
Sodium fumarate	100	84	44	100
Sodium gluconate	100	0	90	0
Sodium malate	100	98	100	100
Sodium octanoate	74	0	0	0

Table 2—continued

	<i>N. amarae</i>	<i>N. asteroides</i> *	<i>N. brasiliensis</i> *	<i>N. otitidis-caviarum</i> *
Sodium propionate	100	100	100	80
Sodium succinate	100	100	80	100
Growth on sole carbon/nitrogen source:				
Acetamide	53	14	50	0
Serine	0	0	70	0
Growth in the presence of:				
0.0001% (w/v) Crystal violet	100	38	10	50
0.001% (w/v) Crystal violet	100	2	0	10
0.01% (w/v) Phenol	100	86	100	90
0.1% (w/v) Phenol	0	43	30	50
0.2% (w/v) Phenyl ethanol	64	43	50	60
0.3% (w/v) Phenyl ethanol	0	22	20	40
0.01% (w/v) Sodium azide	90	62	30	40
0.02% (w/v) Sodium azide	80	12	0	0
5% (w/v) Sodium chloride	27	84	100	100
7% (w/v) Sodium chloride	0	36	0	80
Susceptibility to 10 i.u. penicillin	100	24	10	30
Enzyme activity:				
Allantoin hydrolysis, weak	69	86	100	100
Allantoin hydrolysis, strong	0	24	70	50
Nitrate reduction	100	98	100	100
<i>p</i> -Nitrophenoloxidase	0	14	0	0
Urea hydrolysis, weak	100	98	100	100
Urea hydrolysis, strong	95	79	100	100
Growth at:				
10 °C	16	26	60	30
40 °C	0	100	90	90
45 °C	0	64	20	60
50 °C	0	31	0	10

No strains produced yellow colonies or *m*-nitrophenoloxidase, degraded adenine, used adonitol, melezitose, raffinose (at 1 g l⁻¹), benzamide, sodium lactate or tartrate (at 0.1 g l⁻¹) as sole carbon source or trimethylene-diamine as sole source of carbon and nitrogen, but they all formed a mycelium, contained lipid LCN-A and grew on glucose, sodium acetate, butyrate and pyruvate (at 0.1 g l⁻¹) as sole carbon source.

* Data from Goodfellow (1971) and Lacey & Goodfellow (1975).

Mycobacterium, *Rhodococcus* and the 'aurantiaca' taxon. The *N. amarae* strains showed their highest similarity to *N. asteroides*, *N. brasiliensis* and *N. otitidis-caviarum* but fell outside the aggregate *Nocardia* cluster in the *D_p* analysis. The numerical findings are, therefore, in agreement with previous studies which indicated that a loose association existed between *N. amarae* N667 and *Nocardia sensu stricto* (Orchard *et al.*, 1977; Orchard & Goodfellow, 1980; Goodfellow & Pirouz, 1982). *Nocardia amarae* can readily be distinguished from established species of *Nocardia* using biochemical and physiological criteria (Table 3; Lechevalier & Lechevalier, 1974).

The close similarity between *Corynebacterium sensu stricto* and *Rhodococcus*, and between *C. bovis* and *C. diphtheriae*, in the *D_p* analysis is a reflection of the small number of positive responses shown by the corynebacteria. It has already been shown (Goodfellow *et al.*, 1978) that when organisms that produce many positive responses are compared with less vigorous strains then pattern differences will be artificially small.

The lipid data are in good agreement with those from previous studies (Lechevalier & Lechevalier, 1974; Lechevalier *et al.*, 1976, 1977). Thus, the *N. amarae* strains contain major amounts of straight-chain, saturated and unsaturated acids and tuberculostearic acid and

Table 3. *Characters with the greatest resolving power for differentiating Nocardia amarae, N. asteroides, N. brasiliensis and N. otitidis-caviarum*

Results show the percentage frequencies of positive characters.

Character	<i>N. amarae</i>	<i>N. asteroides</i>	<i>N. brasiliensis</i>	<i>N. otitidis-caviarum</i>
Colony morphology:				
Colonies white, cream, tan	100	17	0	100
Colonies pink, red, orange	0	77	70	0
Degradation of:				
Elastin	0	0	100	0
Hypoxanthine	0	5	100	90
Tyrosine	0	0	100	0
Xanthine	0	0	0	100
Growth on sole carbon source (at 1 g l ⁻¹):				
Glycerol	100	82	90	10
Inositol	85	2	100	90
Rhamnose	90	36	0	0
Growth on sole carbon source (at 0.1 g l ⁻¹):				
Pimelic acid	100	0	10	0
Sodium gluconate	100	0	90	0
Testosterone	0	82	0	100
Growth in the presence of:				
0.001% (w/v) Crystal violet	100	2	0	10
0.02% (w/v) Sodium azide	80	12	0	0
Growth at 40 °C	0	100	90	90

Fig. 2. Two-dimensional t.l.c. of whole-organism acid methanolysate of *N. amarae* N667. 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; M, mycolic acid methyl esters; N, possible long-chain alcohol ('nocardol'); ?, unknown components.

Table 4. Percentage fatty acid composition of strains of *Nocardia amarae*

Relative retention time:		0.57	0.77	0.80	0.97	1.00	1.04	1.16	1.19	1.23	1.27	1.40	1.55	1.68	1.80	1.93	2.11
OV-1 (1 m)		14.0	15.6	16.0	17.6	18.0	18.5										
Equivalent chain length*:																	
OV-1 (2 m)		14.0	16.7	16.0	18.7	18.0	18.2										
Equivalent chain length*:																	
Silar 10C (6 m)		14.0	16.1	16.0	18.1	18.0	Me18										
Assignment†																	
<i>Nocardia amarae</i> N667		2.2	7.7	42.5	21.1	19.0	7.5										
N774		3.1	12.5	26.8	21.1	12.1	16.7	0.7	0.3	2.5		1.9	1.3	0.9			
N775		1.9	9.7	38.6	30.4	10.7	8.7										
N776		3.2	12.0	24.8	26.6	12.7	19.4			1.3							
N777		2.2	5.5	44.2	17.4	19.0	11.7			tr		tr					
N778		1.6	7.9	35.3	36.6	7.0	10.0			1.6							
N779		1.1	11.0	39.4	27.4	2.6	18.5			tr		tr					
N780		6.2	8.7	26.5	15.0	17.0	13.2	0.4	1.2	2.0		1.6	1.9	2.1	2.0	1.6	0.5
N781		1.6	10.3	41.6	27.1	1.4	18.1			tr		tr					
N782		2.3	8.2	39.8	28.4	9.7	11.5			tr		tr	tr				
N783		2.0	14.1	36.5	25.3	3.1	19.0			tr		tr	tr				
N784		1.6	9.7	42.7	30.1	2.2	13.7					tr	tr				
N785		2.7	16.9	27.0	28.5	tr	24.9										
N786		1.9	11.2	42.0	16.4	9.3	19.2										
N787		1.4	9.2	36.4	25.6	6.8	19.3			1.4		tr	tr	tr			
N788		1.6	12.4	42.8	28.1	tr	15.1					tr	tr	tr			
N789		1.8	10.7	42.5	23.6	tr	21.4										
N790		1.2	8.9	38.0	24.1	3.4	21.4			tr	3.0						
N791		1.7	10.9	40.1	26.5	tr	18.1	tr		1.1		1.7					

tr, Trace.

* According to Miwa *et al.* (1960).

† 14:0, 16:0 and 18:0 denote tetradecanoic, hexadecanoic and octadecanoic acids; 16:1 and 18:1 denote hexadecenoic and octadecenoic acids; Me18 denotes 10-methyl-octadecanoic (tuberculoheptanoic) acid.

Fig. 3. Mass spectral fragmentation pathways characteristic of mycolic acid methyl esters from strains of *N. amarae*.

Fig. 4. Partial mass spectrum of the mycolic acid methyl esters from *N. amarae* N667; the spectrum was directly copied and is therefore not normalized. Peaks numbered in *italics* in the anhydromycolate region probably correspond to loss of the elements of methanol from the major anhydromycolates and those in the meroaldehyde region are probably dehydration products. The origin of fragments X and Y is explained by reference to the text and Fig. 3.

produce mycolic acids which characteristically have substantial amounts of unsaturated chains in the 2-position. Mass spectral analysis of the mycolic acids from *N. amarae* strains showed that they were similar in overall size to those of nocardiae since they contained 46 to 54 carbons with up to three double bonds (Minnikin & Goodfellow, 1980) with C_{50} and C_{52} as the major components. However, *N. amarae* strains contain major amounts of dihydrogenated menaquinones with nine isoprene units [MK-9(H_2)] and can thereby be

Table 5. Peaks in the mass spectra of methyl mycolates of *Nocardia amarae* strains N667, N774–N791 (excluding N786) corresponding to anhydromycolates

The main component is denoted by + + + +, any component greater than 50 % of the main peak by + +, and all other significant components by +.

		Classification of <i>Nocardia amarae</i>																	
No. of carbons in parent mycolic acid	No. of double bonds	N667	N774	N775	N776	N777	N778	N779	N780	N781	N782	N783	N784	N785	N787	N788	N789	N790	N791
C ₄₆	682 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	684 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	698 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	700 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C ₄₇	710 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	712 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	714 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	724 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C ₄₉	726 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	728 1	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+
	738 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	740 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C ₅₀	742 1	+	.	.	+	+	.	.	+	+	+	.	+	+	+	+	+	+	+
	752 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	754 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	756 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C ₅₁	758 0	+	+	+	+	+	+	+	+	.	.	+	.	.	+	.	+	+	.
	766 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	768 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	770 1	+	.	.	.	+	.	.	+	+	+	+	+	+	+	+	+	+	+
C ₅₂	780 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	782 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	784 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	786 0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C ₅₃	794 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	796 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	798 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 6. *Peaks in the mass spectrum of methyl mycolates of Nocardia amarae strain N786 corresponding to anhydromycolates*

The main component is denoted by + + +, any component greater than 50% of the main peak by + +, and all other significant components by +.

No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds	Relative intensity	No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds	Relative intensity
C ₄₈	710	3	+	C ₅₆	822	3	++
	712	2	+		824	2	++
C ₅₀	738	3	++		826	1	++
	740	2	++	C ₅₇	836	3	++
	742	1	+		838	2	+
	744	0	+		840	1	+
C ₅₂	766	3	++	C ₅₈	850	3	++
	768	2	++		852	2	++
	770	1	++		854	1	+
	772	0	++	C ₅₉	864	3	+
C ₅₃	780	3	+		866	2	+
	782	2	+		868	1	+
	784	1	+	C ₆₀	878	3	+
C ₅₄	794	3	++		880	2	+
	796	2	++	C ₆₁	892	3	+
	798	1	+++		894	2	+
C ₅₅	808	3	+		896	1	+
	810	2	+	C ₆₂	906	3	+
	812	1	+		908	2	+

Table 7. *Percentage composition of fatty acid methyl esters observed on pyrolysis gas chromatography of methyl mycolates of strains of Nocardia amarae*

Equivalent chain length*: OV-1 (2 m)	14.0	15.5	16.0	15.6	16.6	18.0	17.6
Equivalent chain length*: Silar 10C (6 m)	14.0	15.4	16.0	16.7	17.7	18.0	18.7
Assignment†	14:0	—	16:0	16:1	17:1	18:0	18:1
<i>Nocardia amarae</i> N667	—	—	15.7	31.5	0.8	10.1	41.8
N774	0.4	—	8.8	37.2	1.0	2.2	50.3
N775	—	—	8.9	41.8	tr	2.2	47.1
N776	—	—	13.7	30.7	1.8	4.3	49.5
N777	—	—	11.5	46.2	tr	1.8	40.5
N778	—	—	6.6	36.9	0.9	2.2	53.4
N779	0.3	—	9.1	47.3	1.6	2.8	39.0
N780	0.3	—	26.2	34.5	1.6	8.5	28.9
N781	0.7	—	25.2	18.0	2.6	4.1	49.4
N782	0.4	—	9.0	49.0	1.3	2.3	38.0
N783	0.2	—	8.1	49.8	3.6	3.0	35.4
N784	1.0	—	28.5	19.3	3.5	3.4	44.2
N785	tr	—	22.7	18.0	6.4	4.1	48.8
N786	0.5	—	46.2	1.4	2.6	37.4	11.9
N787	0.4	4.4	11.5	46.7	2.5	3.3	31.2
N788	0.8	—	22.7	21.9	1.4	3.7	49.5
N789	—	—	23.3	18.3	—	4.0	54.5
N790	0.9	—	9.8	50.6	3.2	2.0	33.5
N791	0.3	—	22.7	20.3	2.3	2.7	51.8

tr, Trace.

* According to Miwa *et al.* (1960).

‡ 14:0, 16:0 and 18:0 denote methyl esters of tetradecanoic, hexadecanoic and octadecanoic acids; 16:1, 17:1 and 18:1 denote methyl esters of hexadecenoic, heptadecenoic and octadecenoic acids.

Table 8. Peaks corresponding to molecular ions in the mass spectra of menaquinones isolated from strains of *Nocardia amarae*

The main component of each series is denoted + + +, any components greater than 50% of the main peak by + +, and all other significant components by +.

Menaquinone isoprenologue	MK-7	MK-8		MK-9	
		H ₂ 650	H ₂ 718	H ₂ 786	H ₂ 786
<i>Nocardia amarae</i> N667	+	+	++	+	+++
N774	+	—	+	+	+++
N775	+	—	+	+	+++
N776	+	+	++	+	+++
N777	+	+	++	+	+++
N778	+	+	++	+	+++
N779	+	+	+	+	+++
N780	+	+	+	+	+++
N781	+	+	+	+	+++
N782	+	—	+	++	+++
N783	+	—	+	+	+++
N784	+	—	+	+	+++
N785	+	—	+	+	+++
N786	+	+	+	+	+++
N787	+	+	++	+	+++
N788	+	—	+	+	+++
N789	+	—	+	+	+++
N790	+	—	+	+	+++
N791	+	—	+	+	+++

distinguished from other nocardiae, which possess MK-8(H₄) as the major isoprenologue, but not from mycobacteria or all rhodococci (Collins *et al.*, 1977; Yamada *et al.*, 1976, 1977). Mycobacteria can easily be distinguished from nocardiae, including *N. amarae*, as their mycolates are insoluble in ethanol/diethyl ether (Hecht & Causey, 1976) and much more complex in structure (Minnikin & Goodfellow, 1980).

Nocardia amarae is clearly a good species which is related to established species of *Nocardia* on the basis of chemical, genetical and numerical phenetic data. Its assignment to *Nocardia sensu stricto* is not, however, unambiguous, as *N. amarae* strains only contain dihydrogenated menaquinones with nine isoprene units, possess mycolic acids which release C₁₆ and C₁₈ monounsaturated fatty acids on pyrolysis, are unable to grow in lysozyme broth (Lechevalier & Lechevalier, 1974), while the type strain is not lysed by nocardiphages (Williams *et al.*, 1980). Further work using powerful methods such as *in vitro* nucleic acid pairing techniques is required to determine whether or not *N. amarae* is a genus in its own right.

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