***Nocardia pinensis* sp. nov., an Actinomycete Found in Activated Sludge Foams in Australia**

By LINDA L. BLACKALL,¹*† J. H. PARLETT,³ A. C. HAYWARD,¹
D. E. MINNIKIN,³ P. F. GREENFIELD² AND ANNE E. HARBERS²

¹Department of Microbiology, and ²Department of Chemical Engineering,

University of Queensland, St. Lucia 4067, Queensland, Australia

³Department of Organic Chemistry, The University, Newcastle upon Tyne NE1 7RU, UK

(Received 8 February 1989; accepted 15 February 1989)

A new nocardioform actinomycete was isolated by filament micromanipulation during the course of a study into foaming in activated sludge plants in Australia. It constitutes the second most prevalent foaming organism in Australia after *Nocardia amarae*. These two foaming organisms can be differentiated morphologically, biochemically and chemotaxonomically. The microscopic appearance of the filaments of the new taxon resembles a pine tree. The filaments are Gram-positive, non-acid-fast, non-motile, non-sheathed and about 0.5–1.0 µm in diameter. On a complex medium, the colonies are orange, opaque, macroscopically dry and friable, microscopically moist and shiny, with a pasty texture and an entire edge. The strains are positive for catalase, oxidase and urease and are oxidative in their metabolism of glucose. No strain could degrade hypoxanthine, xanthine, tyrosine, casein, gelatin or aesculin and none could grow with lysozyme. The strains contain peptidoglycan type A_γ, cell wall type IV, whole cell sugar pattern type A, phospholipid type PII, menaquinones ω-cyclo-MK-8 (H₄), fatty acids comprising straight chain saturated and unsaturated acids and tuberculostearic acid, and mycolic acids with 58–64 carbons containing substantial amounts of unsaturated chains in the 2-position. The name *Nocardia pinensis* has been chosen for the new taxon because of the pine tree like appearance of the organism on microscopy. The type strain, UQM3063, is deposited at the University of Queensland, Department of Microbiology Culture Collection.

INTRODUCTION

The formation of stable, dark, viscous foams or scums on the surfaces of the aeration tank section of activated sludge processes was first reported in 1969 (Anonymous, 1969). The stable foam is grey to brown-cream in colour, quite heavy in consistency, up to 30 cm deep, and watersprays applied to the surface have little effect upon its collapse (Lechevalier, 1975; Pipes, 1978; Dhaliwal, 1979). The build up of scum in an activated sludge plant does not appear to accompany any specific plant design with the exception of the aeration system (Hartley, 1982); however, operational mode (Ferguson, 1980; Pipes, 1978; Hartley, 1982), influent type (Lechevalier, 1975; Pipes, 1978; Dhaliwal, 1979; Nelson, 1979; Tricker & Thorpe, 1979), and specific weather patterns (Lechevalier, 1975) have been cited as precipitating factors. Operational problems have been attributed to the scum, and its added financial cost to the plant is as a result of extra personnel required for scum control and clean-up.

When the scum is examined microscopically, many Gram-positive, branching filamentous bacteria are commonly observed (Lechevalier, 1975). Selective accumulation of the actinomycetes in the foam over that in the mixed liquor is common. The predominant organism associated with foams was isolated and identified as *Nocardia amarae* by Lechevalier &

† Present address: School of Microbiology, University of New South Wales, PO Box 1, Kensington 2033, Australia.

Abbreviations: YG, yeast glucose agar; TYG, tryptone yeast extract agar.

Lechevalier, (1974). This is still regarded as the dominant foaming organism; however, *Rhodococcus rhodochrous* (Lemmer & Kroppenstedt, 1984) and '*Microthrix parvicella*' (Blackbeard *et al.*, 1986; Hart, 1985) are also implicated.

Foaming in activated sludge plants was investigated in Queensland, Australia, and during the course of the study, a unique actinomycete was isolated. It differed from all the previously recognized foaming organisms and was therefore fully characterized. This paper describes the new actinomycete for which the name *Nocardia pinensis* sp. nov. is proposed.

METHODS

Isolation and identification. The strains used in the study are listed in Table 1. All fresh isolates came from Queensland, Australia. Isolation of the actinomycetes was by micromanipulation using the Skerman micromanipulator (Skerman, 1968). Bacterial mycelium was manipulated to either yeast glucose agar (YG) containing (per litre), 10 g yeast extract, 10 g glucose and 15 g agar, or to tryptone yeast extract agar (TYG) containing (per litre), 3 g tryptone, 5 g yeast extract, 5 g glucose and 15 g agar. The pH of all media was adjusted to 7.0 unless otherwise stated and sterilization was at 121 °C for 15 min.

Extraction of cellular DNA was unsuccessful. A summary of the methods used and the possible reasons for the failure to extract DNA are presented in the Discussion.

Inoculum preparation. For all tests, unless otherwise stated, inoculum was grown on solid TYG for 7 d (reference strains) or 21 d (*Nocardia pinensis*) at 28 °C in the dark. Biomass from the plates was vortexed in a bottle containing 5–10 ml sterile distilled water and a few 0.25 mm diameter glass beads and the resulting suspension used as inoculum.

Microcolony and cell morphology. (a) *Light microscopy.* Inoculum for cultures came from 7 or 21 d old TYG plate cultures and suspensions were prepared such that when the cells were inoculated onto the TYG media, well separated cells were visible. Microcolonies of cells were photographed with Kodak Plus X pan ASA 125 black and white film using an Olympus BHS microscope with a BH2-PC phase contrast turret condenser and a PM-10AD photomicrographic system.

(b) *Electron microscopy.* Cells of *N. pinensis* were taken from the surface of TYG plates, negatively stained with 1% (w/v) phosphotungstic acid (pH 6.5) for 20 s and examined by transmission electron microscopy using an Hitachi H-800 electron microscope.

Staining reactions. The Gram reaction (Skerman, 1967; Cruickshank *et al.*, 1975), presence of sudanophilic inclusions (Burdon, 1946), presence of polyphosphate inclusions (Cruickshank *et al.*, 1975) and determination of acid fastness (Cruickshank *et al.*, 1975) were done with all strains with biomass from TYG plates.

Biochemical tests. The test media were incubated at 28 °C and, unless otherwise stated, read at 14 and 21 d for the *N. pinensis* strains and at 7 and 14 d for the remainder of strains.

Acid production from carbohydrates. Tryptone yeast extract medium [0.1% (w/v) tryptone; 0.3% (w/v) yeast extract; 1.5% (w/v) agar] with 5 ml 10% (w/v) bromothymol blue l⁻¹ was the basal medium for these tests. Carbon sources were at 0.5% (w/v). The medium was dispensed into tubes and set as slopes. Semi-solid media with glucose were also used and were stab inoculated. All tests were read at 28 d.

Degradation of macromolecules. Concentrations of the macromolecules were as in Gordon (1967) and the basal medium was TYG. Inoculum was spotted onto the solid media. For aesculin hydrolysis, the method of Sneath (1956) was slightly modified such that the medium included tryptone and yeast extract in place of peptone.

Growth in the presence of inhibitors. Mitomycin C (5 µg ml⁻¹; Sigma), isonicotinic acid hydrazide (isoniazid 200 µg ml⁻¹; Sigma), rifampicin (20 µg ml⁻¹; Sigma) and 5-fluorouracil (20 µg ml⁻¹; Sigma) were incorporated into TYG. All antibiotics except isoniazid were filter-sterilized prior to addition to the cooled, sterile basal medium. Isoniazid was added to the basal medium prior to sterilization. The inoculum was spotted on to the solid media.

Tween hydrolysis. TYG containing 0.01% (w/v) CaCl₂ · 2H₂O was the basal medium for this test, which was based on the method of Sierra (1957). Tweens were incorporated at 1% (w/v) and spot inoculation was used.

Detection of urease. TYG (1 litre) with 1.2 ml of 10% (w/v) phenol red and 10 ml of 20% (w/v) filter sterilized urea was the medium for this test, which was based on the method of Christensen (1946).

Nitrite formation from nitrate. The method of Skerman (1967) was used for all strains and liquid TYG was the basal medium.

Lysozyme resistance. The method of Gordon & Barnett (1977) was used for this test and growth was determined after 7 and 21 d. Ability to grow in glycerol broth without lysozyme was used as a control.

Catalase and oxidase test. The catalase test was carried out by adding a drop of 10 vol. H₂O₂ onto the growth on a TYG plate. Instant emission of gas bubbles was positive. The oxidase test was determined by smearing biomass from a TYG plate onto filter paper impregnated with a drop of the oxidase reagent from a Marion oxidase dropper. A blue black colouration to the reagent in 10–20 s was positive.

Table 1. *Strains used in this study*

The first 12 strains are the new actinomycete whilst the remainder are used as reference strains.

Name	Laboratory no.	Reference/isolation site*	Source†
<i>Nocardia pinensis</i>	UQM3063	Bellbowrie STP	Fresh isolate
<i>Nocardia pinensis</i>	NM41	Bellbowrie STP	Fresh isolate
<i>Nocardia pinensis</i>	NM55b	Bundamba STP	Fresh isolate
<i>Nocardia pinensis</i>	UQM3064	Bundamba STP	Fresh isolate
<i>Nocardia pinensis</i>	NM101	Burleigh Park STP	Fresh isolate
<i>Nocardia pinensis</i>	NM102	Burleigh Park STP	Fresh isolate
<i>Nocardia pinensis</i>	NM109	Yandina STP	Fresh isolate
<i>Nocardia pinensis</i>	NM110b	Bundamba STP	Fresh isolate
<i>Nocardia pinensis</i>	NM118	Hayman Island STP	Fresh isolate
<i>Nocardia pinensis</i>	NM167b	Merrimac STP	Fresh isolate
<i>Nocardia pinensis</i>	NM168b	Loganholme STP	Fresh isolate
<i>Nocardia pinensis</i>	NM170	Nudgee STP	Fresh isolate
<i>Nocardia amarae</i>	UQM2058	Lechevalier & Lechevalier (1974); sewage plant; type strain	ATCC 27808
<i>Nocardia amarae</i>	UQM2810	Lemmer & Kroppenstedt (1984); sewage plant	DSM 43602
<i>Nocardia amarae</i>	NM26	Tingalpa STP	Fresh isolate
<i>Nocardia amarae</i>	NM86	Ferny Hills STP	Fresh isolate
<i>Nocardia amarae</i>	NM206	Brackenridge STP	Fresh isolate
<i>Nocardia amarae</i>	NM296	Townsville Golf Course STP	Fresh isolate
<i>Rhodococcus equi</i>	UQV1092	Equine lung	UQV
<i>Rhodococcus equi</i>	UQV1002	Porcine lymph node	UQV
<i>Rhodococcus rhodochrous</i>	UQM2807	Lemmer & Kroppenstedt (1984); sewage plant	DSM 43583
<i>Rhodococcus rhodochrous</i>	UQM2808	Lemmer & Kroppenstedt (1984); sewage plant	DSM 43601
<i>Gordona aurantiaca</i>	UQM2809	Lemmer & Kroppenstedt (1984); sewage plant	DSM 43599

* STP, sewage treatment plant.

† ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, FRG; UQM, Culture Collection, Department of Microbiology, University of Queensland, Brisbane, Australia; UQV, Culture Collection, Department of Veterinary Pathology and Public Health, University of Queensland, Brisbane, Australia.

Cell wall components. (a) *Amino acids and carbohydrates.* The methods followed were those of Harper & Davis (1979) for amino acids and of Stanek & Roberts (1974) for carbohydrates. For both, TLC on cellulose-coated aluminium sheets (20 × 20 cm) (Merck no. 5552) was used and 5–10 µl volumes of hydrolysates were applied to the sheets.

(b) *Fatty acids, mycolic acids, polar lipids and menaquinones.* The *N. pinensis* strains were grown for 21 d on sterile, 0.45 µm pore size, cellulose acetate membrane filters on the surface of TYG plates. The membranes were lifted from the plates and the cells scraped into sterile 5 ml McCartney bottles and lyophilized without cryoprotection. Lipids were extracted according to Minnikin *et al.* (1984).

For polar lipids, two dimensional TLC on aluminium backed silica gel sheets was used (Merck no. 5554). The first direction solvent was chloroform/methanol/water (60:30:6, v/v) and the second chloroform/acetic acid/methanol/water (40:25:3:6, v/v). Different spray systems were used for the different polar lipids (Dobson *et al.*, 1985).

Menaquinones were isolated by two dimensional TLC (Nahaie *et al.*, 1984) and identified by mass spectrometry (Collins *et al.*, 1977).

Mycolic acid analyses followed the methods of Minnikin *et al.* (1975, 1980) and mass spectrometry of the *tert*-butyldimethylsilyl ethers of the isolated methyl mycolates provided ranges of molecular masses and sizes of the chains in the 2-position (Minnikin *et al.*, 1982).

Whole cell acid methanolysis and isolation by TLC of the fatty acid methyl esters (FAMES) was carried out by the method of Minnikin *et al.* (1980). The FAMES were identified using a Shimadzu GC Mini 2 flame ionization chromatograph fitted with both a 25 m OV-1 fused silica bonded column (Alltech) and a 50 m Silar 10C fused silica non-bonded column (Alltech); the injected sample was split between both columns. The operating temperature was programmed from 100 to 200 °C at 5 °C min⁻¹ and peaks quantified using a Shimadzu CE 1B integrator.

API ZYM determinations. The API ZYM system was used according to the manufacturer's (API) specifications. Nineteen enzymes were tested per gallery; galleries were incubated for 6 h at 28 °C in the dark prior to detection.

Suspensions in sterile distilled water (described above) were used as inocula. *N. pinensis* strains were cultured on TYG media whereas the reference strains were cultured on YG media.

Determination of pigment spectra. *N. pinensis* biomass from 21-d-old TYG plate cultures was harvested into chloroform and ethanol and vigorously shaken to extract the orange pigment. A few drops of 20% (w/v) KOH were added to the ethanol extract to determine the presence of flexirubin-type pigments (Reichenbach *et al.*, 1981). The chloroform extract was scanned from 200 nm to 800 nm using a Beckman DU-8 spectrophotometer.

Growth temperature determinations. Slopes of TYG were inoculated with a drop of bacterial suspension and placed into a variable temperature incubator. The temperatures selected for the incubator ranged from 11 °C to 36 °C. Tubes were examined every 3 d for 21 d.

Determination of growth on different media and under different atmospheres. An extensive range of complex and defined media were evaluated for the ability to support the growth of *N. pinensis*. Incubation of all media was at 28 °C in the dark and growth assessment was made after 21 d. Cultures on TYG were incubated in the light, dark, anaerobically, microaerophilically, in a candle jar and in an atmosphere containing 5% (v/v) CO₂.

RESULTS

Microcolony and cell morphology

Results from the *N. amarae* strains isolated from the activated sludge plants agreed with the description given by Lechevalier & Lechevalier (1974). Filament fragments of *N. pinensis* in activated sludge foams and mixed liquors resembled pine trees and as such could be distinguished from *N. amarae* (Fig. 1). *N. pinensis* strains took 10–21 d to produce colonies that were about 1–2 mm in diameter on TYG; however, the growth rate was variable for no apparent reason. On some occasions the strains produced 2 mm colonies in 9–10 d whilst on others, the same strains took 21 d for colonies of the same size to develop. The *N. pinensis* strains on TYG were orange, opaque, macroscopically dry and friable, microscopically moist and shiny, not adherent to the agar, difficult to emulsify or subculture, circular with an entire edge. The majority of the colonies were approximately 1 mm in diameter; however, large colonies approximately 3–4 mm in diameter also occurred. The strains of *N. pinensis* grew in liquid TYG medium as macroscopically visible, orange coloured colonies in the slightly turbid liquid.

The mycelium of *N. pinensis* did not fragment in undisturbed cultures and secondary branching was rare to absent. Aerial mycelium was not visible to the naked eye; however, short branched and unbranched aerial hyphae could be seen microscopically. No specialized morphological structures have been noted (Figs. 2 and 3).

Examination of microcolonies showed the presence of phase bright spherical regions in the mycelium of *N. pinensis* in terminal and intercalary positions. The Gram-positive, septate mycelia of *N. pinensis* tapered after branch points.

Staining reactions and biochemical tests

The biochemical test results and the staining reactions for the *N. pinensis* strains and for the reference strains are shown in Table 2.

Cell wall components

All *N. pinensis* strains contained arabinose, galactose, glucose and ribose as the cell wall carbohydrates and *meso*-diaminopimelic acid (DAP), alanine and glutamic acid as the cell wall amino acids. The fatty acids present in the strains are shown in Table 3 and the mycolic acids are shown in Table 4.

All the mass spectra of the mycolic acid derivatives contained peaks at *m/z* 381, 355 and 353, in the approximate ratios 1.0, 0.5 and 0.25, respectively. These fragments are due to cleavage between carbons 3 and 4, followed by loss of 58 mass units (2-methylpropane), corresponding to hexadecenyl, tetradecenyl and tetradecenyl chains in the 2-position. If the mycolic acid methyl esters were analysed by pyrolysis gas chromatography (Lechevalier & Lechevalier, 1974; Goodfellow *et al.*, 1982), they would release major amounts of methyl octadecenoate and lesser proportions of methyl hexadecanoate and hexadecenoate.

Strains UQM 3064, NM110b, NM167b and NM41 were found to contain lipoquinones whose mass spectra had intense peaks at *m/z* 187 and 225 consistent with a normal 2-methyl-1,4-

Fig. 1. Mycelial fragments of *N. pinensis* (large arrow) and *N. amarae* (small arrow) in a scum sample from Bundamba Sewage Treatment Plant. Bar, 20 μ m.

Fig. 2. Microcolonies of *N. pinensis* NM41 growing on TYG at 28 °C. (a) 24 h incubation. (b) 3 d incubation. Bars, 10 μ m.

Fig. 3. Microcolony of *N. amarae* isolate NM86 grown on YG at 28 °C for 24 h. Bar, 10 μ m.

Table 2. *Results for the biochemical and staining tests for the strains used in this study*

None of the strains grew in the presence of rifampicin ($20 \mu\text{g ml}^{-1}$), mitomycin-C ($5 \mu\text{g ml}^{-1}$), 5-fluorouracil ($20 \mu\text{g ml}^{-1}$) or lysozyme ($50 \mu\text{g ml}^{-1}$); degraded hypoxanthine, xanthine, tyrosine, casein or gelatin; produced acid from glucose (anaerobic), maltose or mannitol; were acid-fast. All strains produced acid from glycerol; contained polyphosphate and sudanophilic inclusions; grew in glycerol broth; were oxidase and catalase positive; were Gram-positive.

Test	<i>N. amarae</i> *	<i>N. pinensis</i> †
Growth in the presence of:		
Isoniazid ($200 \mu\text{g ml}^{-1}$)	+	—
Lipolysis of:		
Tween 20	+	—
Tween 40	+	—
Tween 60	+	—
Tween 80	+	—
Acid production from:		
Glucose (aerobic)	—	+
Mannose	—	+
Salicin	+	—
Aesculin hydrolysis	+	—
Nitrate $\rightarrow$ Nitrite	+	—

* *N. amarae* strains were UQM2058, UQM2810, NM26 and NM86.

† *N. pinensis* strains were all those listed in Table 1 except that NM55b was omitted from the acid production from glycerol, maltose, mannitol, mannose and salicin tests.

Table 3. *Percentage fatty acid composition of strains of N. pinensis*

Strain	Fatty acid type*						TBS
	16:1	16:0	17:1	17:0	18:1	18:0	
UQM3063	15.72	29.80	2.43	3.56	27.03	8.98	12.47
NM41	18.34	28.85	2.04	1.53	20.44	3.19	25.60
UQM3064	20.29	27.43	0.88	0.99	28.84	3.88	15.57
UQM3064	16.30	26.78	3.16	1.87	29.19	5.00	13.16
NM101	15.23	26.23	5.33	6.43	27.41	6.65	12.71
NM109	15.07	29.16	2.34	2.29	26.03	6.27	18.83
NM110b	17.59	19.92	2.59	2.10	32.36	6.97	12.91
NM118	17.69	30.30	1.37	1.46	26.43	4.90	17.85
NM167b	17.38	24.90	3.30	1.68	22.20	4.21	19.12
NM168b	10.70	21.42	4.37	5.72	20.61	7.88	14.20
NM170	—	40.67	—	15.19	6.47	13.92	23.74

—, None detected.

* 16:0, 17:0 and 18:0 denote hexadecanoic, heptadecanoic and octadecanoic acids; 16:1, 17:1 and 18:1 denote hexadecenoic, heptadecenoic and octadecenoic acids; TBS denotes 10-methyloctadecanoic acid (tuberculoistearic acid).

naphthoquinone ring system (Collins *et al.*, 1977). Molecular ions at m/z 720 corresponding to a $\text{C}_{51}\text{H}_{76}\text{O}_2$ tetrahydrogenated menaquinone with eight isoprene units were observed (Howarth *et al.*, 1986). The mass spectra also contained significant fragments at m/z 584 representing loss of a terminal cyclized 2-isoprene moiety. These results are consistent with good representatives of *Nocardia* (Collins *et al.*, 1977).

Strains UQM3064, NM167b, NM110b, NM41, NM109 and NM118 possessed phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and diphosphatidylglycerol as the polar lipids. These strains are therefore representative of phospholipid group PII (Goodfellow & Lechevalier, 1986). Strains not listed in the tables or the text were not analysed. The lipid chemotaxonomy indicates membership of the genus *Nocardia* (Goodfellow & Lechevalier, 1986).

Table 4. Number of carbons and double bonds in the mycolic acids from the mass spectra of the mycolates of *N. pinensis* strains

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

Carbons* . . .	58				59			60		61		62			63			64	
DB† . . .	2	3	4	5	3	4	5	4	5	4	5	4	5	6	4	5	6	5	6
Strain																			
UQM3064		+	+	+				+	+	+	+		+	+	+	+	+	+	+
UQM3064		+	+	+		+	+	+	+	+	+	+	+	+		+	+	+	+
NM167b	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+
NM110b	+	+	+	+				+	+	+	+	+	+	+		+	+	+	+
NM41	+	+	+					+	+	+	+	+	+	+		+		+	+
NM109		+	+					+	+	+	+	+	+	+		+		+	+

* Number of carbons in the parent mycolic acid. † Number of double bonds in the mycolic acid.

Table 5. API ZYM assay results for actinomycetes

Strain*	API enzyme number†																			
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
<i>Nocardia pinensis</i>	—	+	+	—	+	—	±	—	—	+	+	—	—	—	+	—	—	+	—	
<i>Nocardia amarae</i>	+	+	+	+	+	—	+	—	—	+	+	—	—	—	+	+	—	—	—	
<i>Rhodococcus rhodochrous</i>	±	+	+	±	+	+	±	—	—	+	+	—	—	—	+	+	—	—	—	
' <i>Gordona aurantiaca</i> '	+	+	+	+	+	—	—	—	—	+	+	—	—	—	+	+	—	+	—	
<i>Corynebacterium equi</i>	+	+	+	—	+	+	+	—	—	+	+	—	—	—	+	+	—	—	—	

* The strains of *N. pinensis* used were all those listed in Table 1; strains UQM3063 and NM170 were duplicated and gave identical results. For *N. amarae*, the strains were UQM2810, NM26, NM86, NM206, NM296; for *R. rhodochrous*, UQM2807, UQM2808; and for *C. equi*, UQV 1002 and UQV1092.

† Enzymes were: 1, control; 2, phosphatase alkaline; 3, esterase (C4); 4, esterase lipase (C8); 5, lipase (C14); 6, leucine arylamidase; 7, valine arylamidase; 8, cystine arylamidase; 9, trypsin; 10, chymotrypsin; 11, phosphatase acid; 12, phosphoamidase; 13, α -galactosidase; 14, β -galactosidase; 15, β -glucuronidase; 16, α -glucosidase; 17, β -glucosidase; 18, *N*-acetyl- β -glucosaminidase; 19, α -mannosidase; 20, α -fucosidase.

API ZYM

The results for the API ZYM assays are shown in Table 5. The *N. pinensis* strains gave uniform results for the enzymes and were positive for esterase, esterase lipase, leucine arylamidase, acid phosphatase, phosphoamidase, α -glucosidase and α -mannosidase. Major variations in the results between *N. pinensis* and *N. amarae* were in the enzymes β -glucosidase and α -mannosidase. The *N. amarae* strains gave strong positive results for β -glucosidase but were negative for α -mannosidase. Although both groups were positive for acid phosphatase and phosphoamidase, the *N. amarae* strains were consistently strongly positive whilst the *N. pinensis* strains were weakly positive. The results for the reference *Corynebacterium equi* strains agreed with published results (Mutimer & Woolcock, 1982).

Pigment spectra, growth temperature determinations and ability to grow on different media

The orange pigments extracted from *N. pinensis* absorbed maximally at 470 nm. They were not flexirubin-type pigments according to the reaction with 20% (w/v) KOH. It was concluded that the pigment of *N. pinensis* was carotenoid in nature because of its absorption spectrum (Stanier *et al.*, 1977).

The minimum growth temperature for *N. pinensis* was 15 °C, the maximum was 31 °C with optimum growth between 18 and 25 °C.

Strains of *N. pinensis* could not grow on Sabouraud's dextrose agar, malt agar plus 0.5% glucose, corn meal agar plus 0.5% glucose, corn meal agar plus 1% Tween 80 'Microthrix'

medium (Slijkhuis, 1983). Scant growth was recorded for *N. pinensis* on medium I of van Veen *et al.* (1982). These strains grew on oatmeal agar, tyrosine agar, peptone yeast extract iron agar, blood agar, chocolate agar, inorganic salts starch agar, GSP medium (Trick & Lingens, 1984), surfactant medium (Zajic *et al.*, 1977) and medium M69 (Scheff *et al.*, 1984). Good growth was recorded for *N. pinensis* on TYG, isosensitest agar, medium 2 of Gledhill & Casida (1969), trypticase soy agar, yeast malt extract agar, PPLO agar and glycerol asparagine agar base plus 1% glycerol. Isosensitest agar was an Oxoid product whilst the remaining media listed above without references were Difco Bacto products.

There was no difference in the amount of growth or the pigment of the cultures on TYG incubated in the light or dark and strains grew as well in the air as in the CO₂ amended atmosphere. The cultures failed to grow anaerobically, microaerophilically or in the candle jar,

DISCUSSION

N. pinensis is morphologically, biochemically, and in chemotaxonomic properties, different from *N. amarae*, but it is found in predominance in foams on the surface of aeration tanks at activated sludge plants, a feature which it has in common with *N. amarae*. The microcolonies of *N. pinensis* and *N. amarae* showed differences in mycelial morphology (Figs 2 and 3). Filaments of *N. pinensis* contained vesicles; this is not uncommon in actinomycetes and is thought to be a characteristic cellular reaction to age or toxicity of the medium constituents (Goodfellow & Cross, 1984). TYG, being a complex medium, may contain toxic constituents which could vary between medium batches thus affecting the growth rate of *N. pinensis*.

Media that supported the growth of many nocardiae and those used in the identification of these organisms were generally unsuitable as either growth media or as identification media for *N. pinensis*. TYG, the first medium found to support the growth of *N. pinensis*, was used as the basal medium for many of the biochemical tests. Its possible toxic properties may explain some of the negative results obtained for the biochemical tests. *N. pinensis* grew best on a medium containing glycerol as carbon source and asparagine as nitrogen source and this feature is in common with the mycobacteria (Ratledge, 1982); however, the reason for this preference is unknown. It is postulated that glycerol uptake is passive and not carrier mediated.

Using the conventional identification methods of Gordon (1967), *N. amarae* produces acid from glucose (aerobic), glycerol, maltose, mannitol, mannose and salicin (Goodfellow *et al.*, 1982); however, these are not the results obtained when the methods described in the present paper are used (Table 2). The basal medium for the acid production from carbohydrates tests was modified to facilitate the growth of *N. pinensis*. The results obtained using these latter methods show differences between *N. amarae* and *N. pinensis* (Table 2) and that the metabolism of *N. pinensis* is oxidative. The inclusion of complex ingredients in the test medium (tryptone and yeast extract) could be contributing to the different results with *N. amarae*. A defined basal medium that supports the growth of *N. pinensis* is required to determine acid production from carbohydrates.

Attempts to extract the cellular DNA from *N. pinensis* failed as the actinomycetes could not be lysed. Classical actinomycete lysis methods (Mordarski *et al.*, 1976; Marshall *et al.*, 1981) were unsuccessful. Modification to these protocols included extension of the time, and manipulation of the temperatures of exposure, to various key lysis ingredients. The cells of *N. amarae* were successfully lysed. Lysis of many different bacteria can be difficult, particularly within the actinomycetes. *N. pinensis* appears to be especially difficult; however, new methods and enzymes for cell lysis are becoming available all the time and hence these need to be evaluated with this organism. The extreme resistance to lysis highlights another difference between *N. pinensis* and other nocardiae.

Both *N. amarae* and *N. pinensis* are slightly different to most other nocardiae in that their mycolic acids have major amounts of mono-unsaturation in the 2-branch. The type strains of *N. carnea* and *N. vaccinii* share this property (Lechevalier & Lechevalier, 1974; Goodfellow & Lechevalier, 1986). Furthermore, the mycolic acids of *N. pinensis* are slightly longer (58–64 carbons) than those reported for the genus *Nocardia* (44–60 carbons; Goodfellow & Lechevalier, 1986).

Recent studies (Howarth *et al.*, 1986; Collins *et al.*, 1987) have shown that the menaquinones from strains of *Nocardia* have an unusual structure with cyclization of the two terminal isoprene units. These menaquinones, II, III-tetrahydro- ω -(2,6,6-trimethylcyclohex-2-enylmethyl)-menaquinone-6, abbreviated to ω -cyclo-MK-8(H₄), contain eight isoprene units, two hydrogenated (H₄) and the terminal (ω) pair cyclized. The characteristic mass spectral peak at m/z 584, found for the menaquinones from *N. pinensis* and other members of the genus *Nocardia* (Collins *et al.*, 1977), is explained by the loss of the ω -cyclized pair of isoprene units (Howarth *et al.*, 1986).

Although Goodfellow & Cross (1984) and Goodfellow & Lechevalier (1986) give differing descriptions for the term 'nocardioform', the description of *N. pinensis* fits both. This new organism contains mycolic acids and therefore must belong in one of the following taxa: *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Corynebacterium*, *Caseobacter* or the 'aurantiaca' taxon. A combination of the following chemotaxonomic and morphological features was used to align the new strains with *Nocardia* rather than the other mycolic acid containing taxa: size of mycolic acids, presence of tuberculostearic acid, phosphatidylethanolamine, the very characteristic menaquinones (ω -cyclo-MK-8(H₄)), presence of aerial mycelium and relatively slow rate of mycelial fragmentation. The reasons for its inclusion in *Nocardia* rather than the close relative *Rhodococcus* are as follows.

The mycolic acids of *N. pinensis* are slightly longer than those of 'true' nocardiae and the presence of major amounts of unsaturation in the 2-branch is not 'characteristic' for either *Nocardia* or *Rhodococcus*. However, of the nine species of *Nocardia* listed in Bergey's Manual of Systematic Bacteriology (Goodfellow & Lechevalier, 1986), three have this attribute whilst this is not reported in *Rhodococcus*. Furthermore, although it is true that susceptibility to lysozyme (50 $\mu\text{g ml}^{-1}$), 5-fluorouracil (20 $\mu\text{g ml}^{-1}$) and mitomycin-C (5 $\mu\text{g ml}^{-1}$) are features shared by some species of *Rhodococcus* but not by some species of *Nocardia* (Tsukamura, 1981*a, b*; Gordon & Barnett, 1977), they are characters of *N. amarae*. Isoniazid resistance (20 $\mu\text{g ml}^{-1}$) is a property shared by some 34 rhodococci and 10 nocardiae (Orlean *et al.*, 1978); furthermore, this is a property of *N. amarae*. None of the *N. pinensis* strains showed isoniazid resistance. Rifampicin resistance (20 $\mu\text{g ml}^{-1}$) was reported for 225 of 230 'true' nocardiae (Gordon & Barnett, 1977). This feature is not characteristic for either *N. amarae* or *N. pinensis*.

There have been problems in accepting *N. amarae* as a 'true' *Nocardia* (Goodfellow *et al.*, 1982; Goodfellow & Cross, 1984; Goodfellow & Lechevalier, 1986) and the antimicrobial sensitivity patterns reported for *N. amarae* in this paper add to that dilemma as they do not fit those obtained for 'true' nocardiae.

The biochemical, morphological and chemotaxonomic data obtained for the *N. pinensis* strains do not unequivocally align them with *Nocardia* or *Rhodococcus*. However, because there are obvious parallels and similarities between our novel strains and *N. amarae* and because there are more chemotaxonomic and morphological attributes shared by the new strains and *Nocardia* (*vide supra*) than are common to them and *Rhodococcus*, we have aligned the strains with the genus *Nocardia*. Atypical nocardial features are the unusual mycolic acids, the antimicrobial sensitivity patterns, the inability to be lysed by conventional actinomycete lysis methods and the inability to be identified by the conventional methods.

The separation between *N. amarae* and *N. pinensis* is easily facilitated by API ZYM profile comparison, nutritional requirements, menaquinone type, size of mycolic acids and microscopic and macroscopic appearance of the filaments in the sewage plant and on laboratory media. *N. pinensis* can be separated from *N. vaccinii* and *N. carnea* on the basis of nutritional requirements, length of mycolic acids, lysozyme sensitivity and urease production.

N. pinensis cannot be identified using the classical media and methods for biochemical characterization of *Nocardia* (Gordon *et al.*, 1974, 1978; Lechevalier & Lechevalier, 1974), indicating that it is a new taxon. Therefore, on the basis of chemical and biological evidence, the organism that was isolated by micromanipulation from the foams and mixed liquors of activated sludge sewage treatment plants in Australia is described as a new species of *Nocardia*.

Description of Nocardia pinensis sp. nov.

The name *Nocardia pinensis* sp. nov. (M.L. n. *Pinus* genus of pine trees; M.L. adj. *pinensis* pertaining to pines and specifically pine tree like in microscopic morphology) is proposed. The

type strain is UQM3063 deposited in the University of Queensland, Department of Microbiology Culture Collection.

In an activated sludge plant where the organism may proliferate as an extensive surface scum or foam, the microscopic appearance of the filaments resembles a pine tree. The filaments are Gram-positive, non-acid-fast, non-motile, non-sheathed and about 0.5–1.0 µm in width. In a Gram stained preparation using acetone as decolourizer, the filaments appear blue as compared to *N. amarae*, which is dark blue or black. Cells from the activated sludge plant or from artificial culture media contain intracellular sudanophilic and polyphosphate inclusions. On TYG solid medium the strains may take 10–21 d to produce colonies that are about 1–2 mm in diameter, with the growth rate being variable. The colonies are orange, opaque, macroscopically dry and friable, microscopically moist and shiny, with a pasty texture, not adherent to the agar, difficult to emulsify or subculture, circular with an entire edge. When picked from the agar surface, the whole colony can be removed intact. The majority of the colonies are about 1 mm in diameter; however, some colonies that are 3–4 mm in diameter occur. Good growth occurs on a defined medium containing glycerol as a carbon source and asparagine as a nitrogen source. In TYG liquid medium the organisms grow as macroscopically visible colonies in a slightly turbid liquid. The strains are positive for catalase, oxidase and urease, and their metabolism is oxidative. Maximum and minimum growth temperatures are 31 °C and 15 °C, respectively, whilst the optimum lies between 18 and 25 °C. Strains do not degrade hypoxanthine, xanthine, tyrosine, casein, gelatin or aesculin, reduce nitrate or hydrolyse the Tweens 20, 40, 60 or 80 when the basal medium is TYG. Strains do not grow in the presence of rifampicin (20 µg ml⁻¹), mitomycin-C (5 µg ml⁻¹), 5-fluorouracil (20 µg ml⁻¹) or isoniazid (200 µg ml⁻¹) with TYG as basal medium; or in the presence of lysozyme (50 µg ml⁻¹) using glycerol broth as basal medium. No growth occurs anaerobically, microaerophilically or in a candle jar; and growth is not enhanced in an atmosphere of 5% CO₂. Based upon chemotaxonomy, the strains contain peptidoglycan type A1γ, cell wall type IV, whole cell sugar pattern type A, phospholipid type PII, menaquinones ω-cyclo-MK-8(H₄), fatty acids comprising straight chain saturated and unsaturated acids and tuberculostearic acid, and mycolic acids with 58–64 carbons containing substantial amounts of unsaturated chains in the 2-position. Using the API ZYM testing protocol the strains contain esterase (C4), esterase lipase (C8), leucine arylamidase, phosphatase acid, phosphoamidase, α-glucosidase and α-mannosidase.

We wish to thank P. Kelly and S. H. Addison for mass spectroscopy and Ms J. Westcott for operation of the electron microscope.

REFERENCES

- ANONYMOUS (1969). Milwaukee mystery: unusual operating problem develops. *Water and Sewage Works* **116**, 213.
- BLACKBEARD, J. R., EKAMA, G. A. & MARAIS, G. V. R. (1986). A survey of filamentous bulking and foaming in activated sludge plants in South Africa. *Water Pollution Control* **85**, 90–100.
- BURDON, K. L. (1946). Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *Journal of Bacteriology* **52**, 665–678.
- CHRISTENSEN, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *Journal of Bacteriology* **52**, 461–466.
- COLLINS, M. D., PIROUZ, T., GOODFELLOW, M. & MINNIKIN, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *Journal of General Microbiology* **100**, 221–230.
- COLLINS, M. D., HOWARTH, O. W., GRUND, E. & KROPPESTEDT, R. M. (1987). Isolation and structural determination of new members of the vitamin K₂ series in *Nocardia brasiliensis*. *FEMS Microbiology Letters* **41**, 35–39.
- CRUICKSHANK, R., DUGUID, J. P., MARMION, B. P. & SWAIN, R. H. A. (1975). Staining methods. In *Medical Microbiology*, vol. II, pp. 31–57. New York: Churchill Livingstone.
- DHALIWAL, B. S. (1979). *Nocardia amarae* and activated sludge foaming. *Journal Water Pollution Control Federation* **51**, 344–350.
- DOBSON, G., MINNIKIN, D. E., MINNIKIN, S. M., PARLETT, J. H. & GOODFELLOW, M. (1985). Systematic analysis of complex mycobacterial lipids. In *Chemical Methods in Bacterial Systematics*, pp. 237–265. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- FERGUSON, E. H. (1980). Actinomycetes of sewage treatment plants. Paper presented to the Queensland Branch of the Australian Water and Wastewater Association, 7 pp.
- GLEDHILL, W. E. & CASIDA, L. E. (1969). Predominant catalase-negative soil bacteria. III. *Agromyces*, gen. n., microorganisms intermediary to *Actinomyces* and *Nocardia*. *Applied Microbiology* **18**, 340–349.
- GOODFELLOW, M. & CROSS, T. (1984). Classification. In *The Biology of the Actinomycetes*, pp. 7–164.

- Edited by M. Goodfellow, M. Mordarski & S. T. Williams. London: Academic Press.
- GOODFELLOW, M. & LECHEVALIER, M. P. (1986). The genus *Nocardia* Trevisan 1889, 9^{AL}. In *Bergey's Manual of Systematic Bacteriology* vol. 2, pp. 1459–1471. Edited by P. H. A. Sneath. Baltimore: Williams & Wilkins.
- GOODFELLOW, M., MINNIKIN, D. E., TODD, C., ALDERSON, G., MINNIKIN, S. M. & COLLINS, M. D. (1982). Numerical and chemical classification of *Nocardia amarae*. *Journal of General Microbiology* **128**, 1283–1297.
- GORDON, R. E. (1967). The taxonomy of bacteria. In *The Ecology of Soil Bacteria*, pp. 293–321. Edited by T. Gray, R. G. Parkinson & B. Parkinson. Liverpool: Liverpool University Press.
- GORDON, R. E. & BARNETT, D. A. (1977). Resistance to rifampin and lysozyme of strains of some species of *Mycobacterium* and *Nocardia* as a taxonomic tool. *International Journal of Systematic Bacteriology* **27**, 176–178.
- GORDON, R. E., BARNETT, D. A., HANDERHAN, J. E. & PANG, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica* and the nocardin strain. *International Journal of Systematic Bacteriology* **24**, 54–63.
- GORDON, R. E., MISHRA, S. K. & BARNETT, D. A. (1978). Some bits and pieces of the genus *Nocardia*: *N. carnea*, *N. vaccinii*, *N. transvalensis*, *N. orientalis* and *N. aerocolonigenes*. *Journal of General Microbiology* **109**, 69–78.
- HARPER, J. J. & DAVIS, G. H. G. (1979). Two-dimensional thin-layer chromatography for amino acid analysis of bacterial cell walls. *International Journal of Systematic Bacteriology* **29**, 56–58.
- HART, M. A. (1985). Scum formation in a nutrient removing activated sludge plant. *Water South Africa* **11**, 171–178.
- HARTLEY, K. J. (1982). *Nocardia* in activated sludge plants. Paper presented to 1982 Queensland Local Authority Engineers Conference Proceedings, pp. 109–132.
- HOWARTH, O. W., GRUND, E., KROPPESTEDT, R. M. & COLLINS, M. D. (1986). Structural determination of a new naturally occurring cyclic vitamin K. *Biochemical and Biophysical Research Communications* **140**, 916–923.
- LECHEVALIER, H. A. (1975). Actinomycetes of sewage treatment plants. US Dept. of Commerce National Technical Information Service Report no. PB 245914.
- LECHEVALIER, M. P. & LECHEVALIER, H. A. (1974). *Nocardia amarae* sp. nov., an actinomycete common in foaming activated sludge. *International Journal of Systematic Bacteriology* **24**, 278–288.
- LEMMER, H. & KROPPESTEDT, R. M. (1984). Chemotaxonomy and physiology of some actinomycetes isolated from scumming activated sludge. *Systematic and Applied Microbiology* **5**, 124–135.
- MARSHALL, R. B., WILTON, B. E. & ROBINSON, A. J. (1981). Identification of *Leptospira* serovars by restriction endonuclease analysis. *Journal of Medical Microbiology* **14**, 163–166.
- MINNIKIN, D. E., ALSHAMAONY, L. & GOODFELLOW, M. (1975). Differentiation of *Mycobacterium*, *Nocardia* and related taxa by thin-layer chromatographic analysis of whole-organism methanolsates. *Journal of General Microbiology* **88**, 200–204.
- MINNIKIN, D. E., HUTCHINSON, L. G., CALDICOTT, A. B. & GOODFELLOW, M. (1980). Thin-layer chromatography of methanolsates of mycolic acid-containing bacteria. *Journal of Chromatography* **188**, 221–233.
- MINNIKIN, D. E., MINNIKIN, S. M. & GOODFELLOW, M. (1982). The oxygenated mycolic acids of *Mycobacterium fortuitum*, *M. farcinogenes* and *M. senegalense*. *Biochimica et biophysica acta* **712**, 616–620.
- MINNIKIN, D. E., O'DONNELL, A. G., GOODFELLOW, M., ALDERSON, G., ATHALYE, M., SCHAAL, A. & PARLETT, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *Journal of Microbiological Methods* **2**, 233–241.
- MORDARSKI, M., SZYBA, K., PULVERER, G. & GOODFELLOW, M. (1976). Deoxyribonucleic acid reassociation in the classification of the 'rhodochrous' complex and allied taxa. *Journal of General Microbiology* **94**, 235–245.
- MUTIMER, M. D. & WOOLCOCK, J. B. (1982). API ZYM for identification of *Corynebacterium equi*. *Zentralblatt für Bakteriologie und Hygiene, Abteilung 1, Originale C* **3**, 410–415.
- NAHAIE, M. R., GOODFELLOW, M., MINNIKIN, D. E. & HAJEK, V. (1984). Polar lipid and isoprenoid quinone composition in the classification of *Staphylococcus*. *Journal of General Microbiology* **130**, 2427–2437.
- NELSON, J. K. (1979). Start-up and operation of Denver's pure oxygen-activated sludge plant. *Journal Water Pollution Control Federation* **51**, 907–917.
- ORLEAN, P. A. B., GOODFELLOW, M. & MINNIKIN, D. E. (1978). Isoniazid susceptibility as a criterion for the differentiation of mycobacterial species from other mycolic acid containing taxa. *International Journal of Systematic Bacteriology* **28**, 194–196.
- PIPES, W. O. (1978). Actinomycete scum production in activated sludge processes. *Journal Water Pollution Control Federation* **50**, 628–634.
- RATLEDGE, C. (1982). Nutrition, growth and metabolism. In *The Biology of the Mycobacteria*, vol. 1, pp. 185–271. Edited by C. Ratledge & J. Stanford. London: Academic Press.
- REICHENBACH, H., KOHL, W. & ACHENBACH, H. (1981). The flexirubin-type pigments, chemosystematically useful compounds. In *The Flavobacterium-Cytophaga Group* (GBF Monograph Series no. 5), pp. 101–108. Edited by H. Reichenbach & O. B. Weeks. Weinheim: Verlag Chemie.
- SCHEFF, G., SALCHER, O. & LINGENS, F. (1984). *Trichococcus flocculiformis* gen. nov. sp. nov. A new gram-positive filamentous bacterium isolated from bulking sludge. *Applied Microbiology and Biotechnology* **19**, 114–119.
- SIERRA, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek* **23**, 15–22.
- SKERMAN, V. B. D. (1967). *A Guide to the Identification of Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.
- SKERMAN, V. B. D. (1968). A new type of micromanipulator and microforge. *Journal of General Microbiology* **54**, 287–297.

- SLIJKHUIS, H. (1983). *Microthrix parvicella*, a filamentous bacterium isolated from activated sludge: cultivation in a chemically defined medium. *Applied and Environmental Microbiology* **46**, 832-839.
- SNEATH, P. H. A. (1956). Cultural and biochemical characteristics of the genus *Chromobacterium*. *Journal of General Microbiology* **15**, 70-98.
- STANECK, J. L. & ROBERTS, G. D. (1974). Simplified approach to the identification of aerobic actinomycetes by thin-layer chromatography. *Applied Microbiology* **28**, 226-231.
- STANIER, R. Y., ADELBERG, E. A. & INGRAHAM, J. L. (1977). *General Microbiology*, 4th edn. London: Macmillan.
- TRICK, I. & LINGENS, F. (1984). Characterization of *Herpetosiphon spec.*-a gliding filamentous bacterium from bulking sludge. *Applied Microbiology and Biotechnology* **19**, 191-198.
- TRICKER, T. D. A. & THORPE, A. H. (1979). The commissioning and operation of Belper Sewage Treatment Works. *Water Pollution Control* **78**, 515-523.
- TSUKAMURA, M. (1981*a*). Differentiation between the genera *Mycobacterium*, *Rhodococcus* and *Nocardia* by susceptibility to 5-fluorouracil. *Journal of General Microbiology* **125**, 205-208.
- TSUKAMURA, M. (1981*b*). Test for susceptibility to mitomycin C as aids for differentiating the genus *Rhodococcus* from the genus *Nocardia*, and for differentiating *Mycobacterium fortuitum* and *Mycobacterium chelonae* from other rapidly growing mycobacteria. *Microbiology & Immunology* **25**, 1197-1199.
- VAN VEEN, W. L., KRUL, J. M. & BULDER, C. J. E. A. (1982). Some growth parameters of *Haliscomenobacter hydrossis* (syn. *Streptothrix hyalina*) - a bacterium occurring in bulking activated sludge. *Water Research* **16**, 531-534.
- ZAJIC, J. E., GUIGNARD, H. & GERSON, D. F. (1977). Emulsifying surface active agents from *Corynebacterium hydrocarboclasters*. *Biotechnology and Bioengineering* **19**, 1285-1301.