International Journal of Systematic Bacteriology (1998), 48, 237–246 Printed in Great Britain

Nocardia crassostreae sp. nov., the causal agent of nocardiosis in Pacific oysters

Carolyn S. Friedman,[1]† Blaine L. Beaman,[2] Jongsik Chun,[3] Michael Goodfellow,[3] Arthur Gee[4] and Ronald P. Hedrick[1]

Author for correspondence: Carolyn S. Friedman. Tel: +1 707 875 2067. Fax: +1 707 875 2009. e-mail: csfriedman@ucdavis.edu

[1,2] Department of Medicine and Epidemiology, School of Veterinary Medicine[1], and Department of Medical Microbiology, School of Medicine[2], University of California, Davis, CA 95616, USA

[3] Department of Microbiology, The Medical School, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

[4] Natural Sciences Division, Pacific Lutheran University, Tacoma, WA 98447, USA

Seven strains of bacteria were isolated from Pacific oysters, *Crassostrea gigas*, with a focal or systemic disease. The strains were aerobic, Gram-positive, acid-fast, produced a mycelium which fragmented into irregular rod-like elements, had a peptidoglycan containing *meso*-diaminopimelic acid, arabinose and galactose as major sugars, mycolic acids with 46–58 carbon atoms and G+C-rich DNA. All of these properties are consistent with the classification of the organisms in the genus *Nocardia*. A partial sequence of the 16S rRNA gene of isolate NB4H was determined following isolation and cloning of the PCR-amplified gene. The sequence was aligned with those of representative mycolic-acid-containing taxa and a phylogenetic tree was generated using the neighbour-joining method. It was evident from the phylogenetic tree that the three strains tested, RB1, OB3P and NB4H, were identical and belonged to the *Nocardia otitidiscaviarum* rRNA sub-group. The biochemical, chemical, morphological and physiological properties of the isolates were also essentially identical and served to distinguish them from representative nocardiae. It is, therefore, proposed that the strains isolated from the diseased Pacific oysters be assigned to a new species, *Nocardia crassostreae*. The type strain is NB4H (= ATCC 700418).

Keywords: *Nocardia crassostreae* sp. nov., nocardiosis, Pacific oysters

INTRODUCTION

The genus *Nocardia* encompasses several species that cause clinical diseases in animals, including humans (10, 11, 30). Nocardiae can be identified with certainty only by laboratory means and their occurrence is almost certainly underestimated. Nevertheless, it is known that nocardiae can infect a large variety of mammals, such as cats (3), and several species of fish such as *Hyphessobycon innesi* (neon tetras), *Oncorhynchus mykiss* (rainbow trout; 39) and *Seriola quinqueradiata* (Japanese yellowtail; 18).

A *Nocardia*-like bacterium was recently isolated from cultured Pacific oysters, *Crassostrea gigas*, which experience summer mortality in several embayments in British Columbia, Canada, and the State of Washington, USA (7, 8). The infected oysters often contained focal areas of brown discolouration on the mantle or raised green or yellow nodules in the adductor muscle, gills, heart and mantle. The nodules were composed of host haemocytes surrounding Gram-positive, acid-fast filamentous bacteria. Initial identification of the bacterium as a member of the genus *Nocardia* was based on morphological and tinctorial characteristics. The disease was, therefore, called Pacific oyster nocardiosis (7).

The recognition of *Gordona amarae* (12, 20, 36) and *Skermania piniformis* (3) as organisms previously classified as nocardiae leaves the genus *Nocardia* as a homogeneous taxon encompassing ten validly described species. Members of the genus form extensively branched, substrate hyphae which fragment into rod-shaped, non-motile elements; aerial hyphae are usually formed but are sometimes only visible microscopically (11, 14, 15). Nocardiae are also characterized by a number of chemical markers, including the presence of *meso*-diaminopimelic acid (*meso*-DAP), arabinose and

† **Present address:** Bodega Marine Laboratory, PO Box 247, Westside Road, Bodega Bay, CA 94923, USA.

Abbreviations: DAP, diaminopimelic acid; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester.

The GenBank/EMBL accession numbers for the sequences reported in this paper are U92799, U92800 and Z37989.

galactose (wall chemotype IV *sensu*, 26), mycolic acids with 40–60 carbon atoms and DNA G+C content of 64–72 mol% (10, 11). Nocardiae fall into two ribosomal sub-groups, one corresponding to *Nocardia asteroides* and related taxa and the other to *Nocardia otitidiscaviarum* and allied species (2). The separation of the two sub-groups is based on differences found in helix 37.1.

In the present study, *Nocardia*-like actinomycetes isolated from oysters grown in several locations in the Pacific Northwest of the USA and British Columbia, Canada, were the subject of a polyphasic taxonomic study designed to determine the relationship of the isolates with representative strains of the genus *Nocardia*.

METHODS

Organisms and culture conditions. The test strains consisted of seven Nocardia-like strains and eight reference strains. The seven bacterial strains obtained from individual oysters from the following locations. Five strains were obtained from two embayments in Southern Puget Sound, WA, USA: RB1, RB13 and RB29 were obtained from Rocky Bay, and strains OB3P and OB5H were obtained from Oakland Bay. Two additional strains were obtained from British Columbia, Canada: strain SI was obtained from Scott Island and the type strain NB4H was obtained from Nanoose Bay, Vancouver Island. Six of the latter were provided by Dr B. L. Beaman, namely *Gordona amarae* strain Se-6, isolated from a sewage treatment plant, and clinical isolates of *N. asteroides* strain GUH-2, *Nocardia brasiliensis* strain 17E, *N. otitidiscaviarum* strain 112, *Mycobacterium paratuberculosis* strain 116 and *Rhodococcus* strain SL. The remaining reference strain, *Nocardia seriolae* strain N1 (formerly '*Nocardia kampachi*'; 24), was donated by Dr R. Kusuda (Department of Cultural Fisheries, Faculty of Agriculture, Kochi University, Kochi, Japan) and *Nocardia farcinica* was obtained from the American Type Culture Collection (ATCC 3318^T). Two additional type strains were obtained from the ATCC: *N. asteroides* (ATCC 19247^T) and *N. seriolae* (ATCC 43993^T). The strains were maintained on Brain Heart Infusion (BHI) agar plates, with or without supplemental NaCl, at room temperature.

Biomass for the chemotaxonomic studies was prepared in flasks of BHI broth shaken at 180 r.p.m. for 5–7 d at 28 °C. Biomass for the 16S rRNA study was cultivated on BHI agar for 2 weeks at 25 °C. At maximum growth, the broth cultures were checked for purity, killed with formaldehyde (1%, v/v), harvested by centrifugation and washed with distilled water.

Morphological and staining properties. Colonial and micromorphological properties of the seven strains isolated from the Pacific oysters and some of the reference strains (Table 1) were observed after 3–5 d growth on BHI agar. The presence of aerial hyphae was determined microscopically. The same preparations were used to determine the staining properties of the organisms using the acid-fast (Kinyoun), Gram and periodate–Schiff base stains (Difco).

Electron microscopy. Colonies (about 1 mm in diameter) of isolate RB29 and *N. asteroides* strain GUH-2 were grown for 5 d at 22 °C. The colonies were fixed in 2·5% (v/v) glutaraldehyde in 0·1 M phosphate buffer (pH 7·4), post-fixed in aqueous osmium tetroxide (1%, w/v), dehydrated through a graded series of acetone concentrations and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and sections were viewed and photographed using a Phillips EM400 electron microscope.

Biochemical characterization. The seven isolates and four reference strains of *Nocardia* (Table 2) were examined for their ability to form acid from D- and L-arabinose, galactose, glucose, *meso*-inositol, lactose, maltose, D-mannitol, sucrose, trehalose and xylose using an inorganic nitrogen basal medium supplemented with 1% (w/v) of the appropriate sugar (14). Bacterial suspensions in 2% saline were used to inoculate test tubes containing the medium prior to incubation at 28 °C for 1 month. Degradation of aesculin (0·1%, w/v), casein (2%, w/v), gelatin (0·4%, w/v), hypoxanthine (2%, w/v), starch (2%, w/v), tyrosine (2%, w/v) and xanthine (2%, w/v) were recorded after incubation at 25 °C for up to 6 weeks using established methods (13, 22). Hydrolysis of Tweens 20 and 80 was detected in Sierra's medium (38), following incubation at 25 °C for up to 1 month. The presence of urease was observed after 14 d growth at 25 °C using Bacto urea base slants (Difco) and urease broth (40). Similarly, resistance to catalase was determined on BHI slants after 14 d.

The ability of the test strains to grow on citrate, creatine, ethanol, gluconic acid and propionic acid (at 1%, w/v) was examined using a carbon-free medium (13). Growth was evaluated after 1 month at 28 °C by comparison with that of strains grown on BHI agar. Similarly, the ability of the strains to use salicin as a sole carbon source was determined as described by Goodfellow (9). The organisms were also examined for their ability to grow on lysine, ornithine and valine (at 1%, w/v) as sole sources of carbon and nitrogen using an established basal medium (13).

Resistance to 5-fluorouracil (20 μg ml^{-1}), isoniazid (200 μg ml^{-1}), mitomycin C (57 μg ml^{-1}) and rifampicin (20 μg ml^{-1}) was recorded following incubation at 25 °C for 1 week with glucose-yeast (both 5%, w/v) agar as basal medium; presence or absence of growth was confirmed following subculture onto BHI agar. Survival at 50 °C was examined by incubating triplicate cultures of each organism in BHI tubes; after 8 h at 50 °C the inoculated tubes were cooled to 23 °C in a waterbath then held at this temperature for up to 1 month. Growth in the tubes was used to subculture onto BHI agar to confirm positive results. The organisms were also examined for their capacity to grow on BHI agar supplemented with sodium chloride (2 and 5%, w/v) following incubation at 25 °C for up to 1 month. Similarly, the strains were observed for their ability to survive at 5 °C and to grow on BHI agar at 22 °C. Survival at 5 °C for 1 month was assessed following passage of bacteria onto fresh BHI agar and incubation at 22 °C. Visible growth after 5–7 d indicated survival.

Detection of isomers of DAP. The procedure of Staneck & Roberts (40) was used to prepare acid hydrolysates from biomass (300 mg wet wt) of *G. amarae* strain Se-6 and the seven isolates from oysters. Aliquots of each sample (2·5 μl) were spotted along the bottom of thin-layer cellulose sheets (Eastman Kodak 13255), and the amino acids in the hydrolysates were separated for 16 h using the solvent system methanol, distilled water, 6 M HCl and pyridine (80:26:4:10, by vol.; 16). The chromatogram was dried in air, sprayed with 0·75% (w/v) ninhydrin in 95% ethanol and heated at 100 °C for 3 min [procedure modified from Hoare & Work (16) and Staneck & Roberts (40)]. The isomers of

DAP were detected following comparison with the results obtained with a standard mixture of DAP isomers (Sigma).

Extraction and characterization of mycolic acids. Mycolic acid methyl esters (MAMEs), prepared from 0·5–1·0 g wet wt bacterial cells of *G. amarae* strain Se-6, *N. asteroides* strain GUH-2, *M. paratuberculosis* strain 116, *Rhodococcus* strain SL and the seven isolates from oysters, using the procedure of Beaman & Moring (1), were spotted onto activated TLC plates and separated in petroleum ether: diethyl ether (4:1, v/v) according to Minnikin *et al.* (31). The MAMEs were visualized using UV illumination (266 nm), scrapped from the TLC plates, extracted three times in lipopure methanol (Allied Associates)/petroleum ether (1:4, v/v) and the preparations were centrifuged at 209 ***g*** for 10 min at 4 °C to pellet any residual silica dust. The supernatants were decanted into clean test tubes, dried under argon, resuspended in 1·0 ml isooctane and analysed by GLC using a procedure modified from Lechevalier *et al.* (29, 30).

The samples were analysed using an Hewlett-Packard 5880A autoinjection gas chromatograph fitted with a flame-ionization detector and a capillary column (0·2 mm × 12 m DB-1 methyl-silicone-fused silica) following a protocol modified from that of Beaman & Moring (1). The initial oven temperature for the capillary column of 230 °C was followed by a 15 °C min^{-1} linear gradient increase to 350 °C. The inlet temperature was 350 °C and the temperature of the flame-ionized detector was 375 °C. The retention time for each fatty acid methyl ester (FAME) and aldehyde pyrolysis product of the MAMEs of each strain examined was compared to that of a fatty acid standard (Applied Sciences Laboratories) or to aldehydes from pyrolysis of *N. asteroides* strain GUH-2 that were previously identified by GLC-MS (1), respectively. Mycolic acid chain-lengths were estimated by combining the chain-lengths of the smallest FAME and aldehyde products to yield the smallest parent MAME possible. The largest MAME was determined in the same manner.

Wall sugar analyses. Biomass (about 100 mg wet wt) prepared from *G. amarae* strain Se-6, *N. asteroides* strain GUH-2 and the seven oyster isolates was hydrolysed at 95 °C for 15 h in 3 ml 2 M H_2SO_4 (33), cooled to 23 °C, centrifuged at 1700 ***g*** for 10 min and passed through a 0·22 μm nylon filter (MicronSep; Magna Nylon Membrane Filters). Protein was precipitated from the samples by the addition of 1:10 (v/v) 60% perchloric acid. The resultant samples were split into two equal fractions for analysis by HPLC and paper chromatography, respectively. Solutions of 2 mg ml^{-1} trehalose, D(−)-arabinose and/or D(+)-galactose in distilled water were used as standards.

Samples (5 μl) were manually injected into an HPLC apparatus fitted with a Waters 501 HPLC pump, a U6K injector and a 300 × 7·8 mm Bio-Rad cation-exchange column with a 9·0 μm particle size. The mobile phase was 0·01 M H_2SO_4 at a rate of 0·6 ml min^{-1} at 41 °C. Absorbance of each fraction at 193 mm was detected using a Kratos Spectroflow spectrophotometer. In the corresponding experiments, approximately 100 μl of the bacterial extracts and standards were spotted onto Whatman no. 3 paper and analysed by descending paper chromatography according to the methods of Murray & Proctor (33). Confirmation that the experimental samples co-migrated with the appropriate standards was determined by elution of parallel unstained fractions with approximately 200–300 μl distilled water followed by HPLC analysis.

Isoprenoid quinone analysis. Menaquinones were extracted from freeze-dried biomass (50 mg) of strain NB4H and *N. asteroides* N317^T using the small-scale procedure of Minnikin *et al.* (32). Purified menaquinones were separated by HPLC, using a Pharmacia LKB instrument fitted with a Spherisorb octaldecylsilane column (5 μm) with acetonitrile/2-propanol (75:25, v/v) as the mobile phase. The menaquinones were detected at 254 nm.

Extraction of DNA. Nucleic acids were extracted from *G. amarae* strain Se-6, *N. asteroides* strain GUH-2, *N. otitidis-caviarum* strain 112, *N. seriolae* strain N-1 and from the seven isolates from oysters using modifications of established procedures (41, 43). The strains were grown, at 28 °C on a rotary shaker at 150 r.p.m., to late-exponential phase in flasks of BHI broth buffered with sodium dihydrogen phosphate (2·75 g l^{-1}). The biomass was harvested by centrifugation at 7096 ***g*** for 20 min at 4 °C and transferred to BHI broth supplemented with glycine (4%, w/v), then shaken at 150 r.p.m. for 48 h at 29 °C. The resultant growth was lysed with lysozyme (mg ml^{-1}) for 90 min at 37 °C and with SDS (5%, w/v) for 15 min at 37 °C (41). Nucleic acids were extracted from the aqueous phase using modifications of the method of Whipple *et al.* (43). The crude nucleic acids were pelleted by centrifugation (151 194 ***g***, 20 min at 4 °C) and the supernatant was mixed with isopropyl alcohol (1:1, v/v) to precipitate any additional nucleic acids. The nucleic acids were pelleted as before, washed in 70% ethanol and then suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA) at pH 8·0. RNA was removed from the preparations by the addition of 0·5 vol. 7·5 M ammonium acetate followed by centrifugation at 9268 ***g*** for 10 min at 4 °C. The supernatant was added to 6 ml 2-propanol and the resultant mixture was centrifuged to pellet the DNA, which was resuspended in TE buffer. Proteinase K (1 mg ml^{-1}) and SDS (1%, w/v) were added to the mixture and incubated at 50 °C for 1 h. The DNA was further extracted with TE-saturated phenol and purified according to methods of Whipple *et al.* (43).

DNA base composition. The G+C content of the DNA, prepared as described above, was determined by a method modified from that of Nelson *et al.* (35). The DNA was heated in the cuvette of a spectrophotometer in 0·1 °C min^{-1} increments from 72–90 °C and absorbance was read at 260 nm.

PCR amplification and sequencing of the 16S rRNA gene. Isolation of the chromosomal DNA, amplification, cloning and sequencing of the 16S rRNA gene of oyster isolates was carried out using the following two methods: (I) small-subunit rRNAs for clinical bacterial isolates RB1, OB3P and NB4H were sequenced with a modified dsDNA template protocol as presented in Protocol p/n 402078-ABI Prism Dye Terminator Cycled Sequencing (Perkin-Elmer). The 16S rRNA gene was amplified from bacterial cells that were washed in an alkali solution and heat-lysed to liberate genomic DNA. PCR was performed using AmpliTaq Gold DNA polymerase (PE/Applied Biosystems). An initial incubation at 95 °C for 10 min was employed to activate the polymerase and increase the specificity of the PCR reaction. The amplification products were purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular mass cut-off membranes. PCR products were checked for quality and quantity by agarose gel electrophoresis before carrying out cycle sequencing.

Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and they were labelled with dideoxy fluorescent dye terminators.

Table 1. Morphological and staining properties of the isolates from Pacific oysters and the reference *Nocardia* strains

Oyster isolates are NB4H, OB3P, OB5H, RB1, RB13, RB29 and S1. +, Positive; −, negative; v, variable; and w, weak response.

Property	Oyster isolates	*N. asteroides* strain GUH-2	*N. brasiliensis* strain 17E	*N. otitidiscaviarum* strain 112	*N. seriolae* strain N-1
Colony colour:					
Beige	−	+	+	+	−
Pale orange	−	−	+[v]	−	+
Pale yellow	+	−	−	−	+
Colony morphology:					
Aerial hyphae	−	+	+	+	−
Filamentous margins	+[w]	+	+	+	+[v]
Growth into agar	+[w]	+[w]	+	+	+[w]
Dry and waxy	+	+/−	+/−	+	+
Raised and convex	−	−	−	−	−
Wrinkled	+	+[v]	[v]	[v]	+
Soluble pigment	−	+	−	−	−
Micromorphology:					
Beaded	+[v]	+[v]	+[v]	+[v]	+[v]
Filamentous	+	+	+	+	+
Fragmentation into rods and cocci	+	+	+	+	+
Staining properties:					
Acid-fast	+	+[w]	+[w]	+[w]	+[w]
Gram	+	+	+	+	+
Periodate–Schiff	+	+	+	+	+

Excess dye-labelled terminators were removed from the sequencing reaction mixture using a Sephadex G-50 spin column. The products were collected by centrifugation, dried and stored at −20 °C. Stored samples were re-suspended in a solution of formamide/blue dextran/EDTA and denatured prior to loading on electrophoresis gels. A 5% Long Ranger (FMC) polyacrylamide/urea gel was used to separate the sequencing reaction products. The gels were run for 6 h and data were analysed with PE/Applied Biosystems DNA editing and assembly software. Sequencing was done by MIDI Labs (Microbial ID).

(II) Small-subunit rRNAs of clinical bacterial isolate NB4H and of the 10 reference *Nocardia* species were sequenced with a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit and an 373A DNA sequencer (both Applied Biosystems) as described previously (2).

Phylogenetic analysis. The sequence editor and analysis program, version 3.0.4, of Gary J. Olsen was used for data entry, editing, sequence alignment, similarity matrix generation and output of tree diagrams (Fig. 3). The program calculates the fractional sequence similarity and Jukes–Cantor distance value for pairs of sequences (17). Tree diagram construction was based on paired distance values. The OB3 sequence was also sent to the Ribosomal Database Project (RDP) for analysis (25). The RDP data (not shown) is consistent with our conclusions.

In a separate trial, the 16S rRNA gene sequence of the oyster isolate NB4H was aligned manually against sequences of representative mycolic-acid-containing actinomycetes using the program AL16S to determine the secondary structure (2). The additional sequence data were obtained from the RDP release 4 (25) and from our own entries. An evolutionary distance matrix was calculated using the Jukes–Cantor algorithm (17). A phylogenetic tree was constructed using the neighbour-joining method (36) with *Arthrobacter globiformis* DSM 20124^T as the outgroup strain. The stability of the resultant tree was determined using the bootstrap method (5). All analyses were carried out using the PHYLIP package (6).

Nucleotide sequence accession numbers. The two almost complete 16S rRNA sequences (1513 nucleotides each) have the accession GenBank numbers U92799 and U92800 for *N. crassostreae* strains RB1 and OB3P, respectively. The partial 16S rRNA gene sequence of the oyster isolate NB4H has the EMBL accession number Z37989.

The accession numbers of the other nucleotide sequences used in this study are M23411 (*Arthrobacter globiformis*), D38575 (*Corynebacterium bovis*), X53185 (*Corynebacterium variabilis*), M59058 (*Corynebacterium xerosis*), M80635 (*G. amarae*), X80634 (*Gordona sputi*), X79286 (*Gordona terrae*), M20940 (*Mycobacterium bovis*), X79094 (*Mycobacterium chlorophenolicum*), X52931 (*Mycobacterium simiae*), Z36934 (*Nocardia asteroides*), Z36935 (*N. brasiliensis*), Z36928 (*Nocardia brevicatena*), Z36929 (*Nocardia carnea*), Z36936 (*N. farcinica*), Z36930 (*Nocardia nova*), M59056 (*N. otitidiscaviarum*), Z36925 (*N. seriolae*), Z36926 (*Nocardia transvalensis*), Z36927 (*Nocardia vaccinii*), X53203 (*Rhodococcus erythropolis*), X79186 (*Rhodococcus fascians*), X77779 (*Rhodococcus globerulus*), Z35435 (*Skermania pinensis*) and Z36933 (*Tsukamurella paurometabola*).

RESULTS

Morphological and staining properties

All seven strains isolated from diseased Pacific oysters were Gram-positive, acid-fast and produced a well-

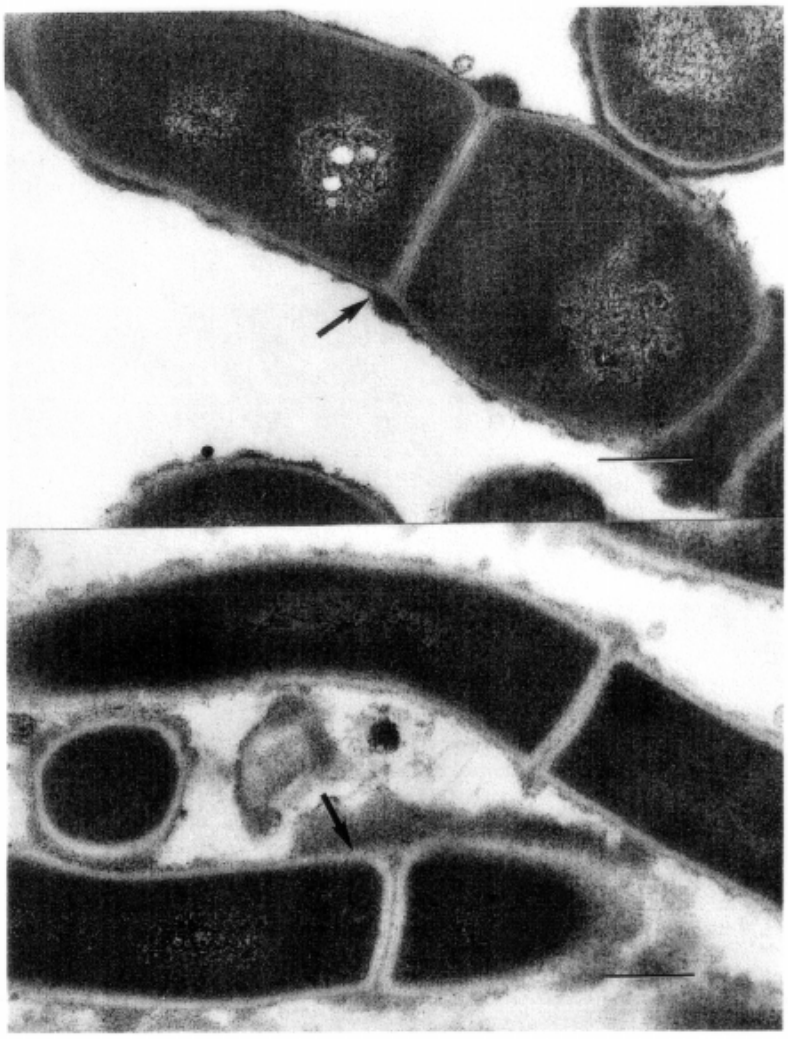

Fig. 1. Transmission electron micrographs showing trilaminar cell wall structure (arrows) in (top) strain RB29, isolated from a diseased Pacific oyster, and (bottom) *N. asteroides* strain GUH-2. Bars, 0·27 μm.

developed mycelium which fragmented into irregular rod-shaped forms. The colonies of these organisms were dry, waxy and wrinkled but did not carry aerial hyphae. The colonial morphology of the isolates differed from that of all of the *Nocardia* reference strains but was most similar to *N. seriolae* (Table 1).

Electron microscopy

Photomicrographs of *N. asteroides* strain GUH-2 and oyster isolate RB29 demonstrated that both strains are emarginated by a trilaminar cell wall (Fig. 1). The electron-dense peptidoglycan layer is separated from the cytoplasm by a less dense area. In addition, the outer wall is also separated from the murein layer by an electron-lucent layer.

Biochemical characterization

The seven strains isolated from the diseased Pacific oysters gave identical results for all of the tests. Both the isolates and the *Nocardia* reference strains were catalase-positive and urease-positive on slants (see Table 2 for urea broth results), hydrolysed aesculin, produced acid from glucose and sucrose, were resistant to lysozyme, grew in the presence of sodium chloride (2%, w/v), survived at 5 °C for 1 month and grew at 22 °C. These strains were unable to hydrolyse gelatin or starch, did not produce acid from D- or L-arabinose, galactose, lactose, maltose, rhamnose or xylose, were unable to use creatine or ethanol as sole carbon sources, or L-arginine and lysine as sole carbon and nitrogen sources, and did not grow at 5 °C. The biochemical and physiological properties which gave differential results are shown in Table 2. The biochemical results of all reference strains were identical to those of the type strain of each nocardial species examined (9, 15, 26).

Chemotaxonomic characterization

Whole-organism hydrolysates of *G. amarae* Se-6 and the seven strains from Pacific oysters contained *meso*-DAP. These organisms and *N. asteroides* strain GUH-2 were found to contain arabinose, galactose and glucose when whole-organism hydrolysates were analysed by HPLC; these sugars were also detected in

Table 2. Some differential phenotypic properties of the isolates from Pacific oysters and the reference *Nocardia* strains

Oyster isolates are NB4H, OB3P, OB5H, RB1, RB13, RB29 and S1. +, Positive; −, negative; v, variable; and w, weak growth relative to that in BHI agar; NT, not tested.

Test	Oyster isolates	*N. asteroides* GUH-2	*N. brasiliensis* 17E	*N. otitidiscaviarum* 112	*N. seriolae* N-1
Acid from:					
D-Mannitol	−	−	+	+	−
Trehalose	+	+	−	+	−
Decomposition of:					
Casein	−	−	+^{w}	−	−
Hypoxanthine	−	−	+	+	−
Tween 20	+^{vw}	−	+	+	+^{w}
Tween 80	+^{vw}	−	−	+	+
Tyrosine	−	−	+	−	−
Urea (broth)	−	+	+	+	−
Xanthine	−	−	−	+	−
Growth at:					
pH 9·0	−	+	+	−	−
37 °C	−	+	+	+	−
Growth after heating (50 °C/8 h)	−	+	−	+	−
Growth with 5% NaCl	−	+	+	+	+
Growth on sole C sources:					
Citrate	−	−	+	−	−
Gluconic acid	−	+	−	−	+^{w}
Propionic acid	−	+	+	+^{w}	−
Salicin	−	+	NT	NT	NT
Growth on sole C and N sources:					
Ornithine	−	−	−	−	+^{w}
L-Valine	−	+	+	+	−
Resistance to:					
5-Fluorouracil	−	+	+	+	NT
Isoniazid	+	+	+	+	−
Mitomycin C	−	+	+	−	+
Rifampicin	−	−	+	+	−

eluents of the appropriate unstained HPLC strips. The HPLC results were confirmed in the corresponding analyses using descending paper chromatography. The major wall sugars detected on the basis of the colorimetric and R_f data were arabinose and galactose. Strain NB4H and *Nocardia asteroides* strain N317^T contained predominant amounts of hexahydrogenated menaquinones with eight isoprene units in which the two end units were cyclized [MK-8(H_4)C].

The DNA G+C content of the test strains was 67·0–71·0 mol%. The values for the seven strains isolated from diseased Pacific oysters was 68·6–69·3 mol%, the mean value being 68·8 mol%. Slightly higher DNA base composition values were recorded for *N. asteroides* strain GUH-2 (70·8 mol%), *N. otitidiscaviarum* strain 112 (70·4 mol%) and *N. seriolae* strain N-1 (69·5 mol%). The corresponding value for the *G. amarae* strain Se-6 was 67·1 mol%.

The test and reference strains contained MAMEs with characteristic mobilities on TLC of the crude methyl mycolate preparations. The R_f value of the MAMEs of *N. asteroides* strain GUH-2 and the seven oyster isolates was 0·246. The corresponding R_f values for *G. amarae* strain Se-6 and *Rhodococcus* strain SL were 0·258 and 0·223, respectively. *M. paratuberculosis* strain 116 produced a multispot mycolate pattern with R_f values of 0·291 and 0·248. The results obtained when purified MAMEs were examined by GLC are shown in Table 3. The oyster pathogens contained mycolic acids with 46–58 carbon atoms, which on pyrolysis gave both odd- and even-numbered methyl mycolates (Fig. 2c, f). Similarly, *N. asteroides* strain GUH-2 contained mycolates with 42–56 carbons and major GLC peaks representing methyl esters with even numbers of carbons, though traces of $C_{13:0}$, $C_{15:0}$ and $C_{17:0}$ were detected. The mycolic acids of *N. seriolae* strain N-1 ranged from approximately C_{48} to C_{60} with pyrolysates primarily composed of even numbers of carbons; the aldehyde products of this strain were longer than those of the other test strains. In all of the pyrolysis profiles mentioned above, the methyl esters were separated from the aldehydes (Fig. 2). This was not the case with *M. paratuberculosis* strain 116 or

Table 3. Relative percentage area of peaks of individual FAMEs and aldehydes from pyrolysis of mycolic acids of the seven strains isolated from diseased Pacific oysters and the reference strains

No. of carbons	Oyster isolate:							Reference strain:	
	NB4H	OB3P	OB5H	RB1	RB13	RB29	SI	*N. asteroides* GUH-2	*N. seriolae* N-1
Aldehydes									
C30:2	0	0	0	0	0	0	0	4	0
C32:2	0	0	TR	0	2	2	0	11	0
C34:4	6	5	4	4	3	5	4	17	0
C36:4	16	16	24	4	12	10	16	18	2
C38:4	29	15	15	30	30	18	23	3	2
C40*	3	16	6	11	6	9	5	0	2
C42*	4	6	8	10	8	8	10	0	4
C44*	0	0	0	0	0	0	0	0	15†
Methyl esters									
C12:0	4	9	5	4	5	3	9	1	17
C13:0	4	8	TR	TR	9	7	4	TR	16
C14:0	20	12	23	20	15	16	15	10	30
C15:0	4	7	TR	TR	5	6	3	TR	11
C16:0	11	7	15	8	7	7	11	27	TR
C17:0	TR	TR	TR	TR	TR	TR	TR	TR	11
C18:0	TR	TR	TR	TR	TR	TR	TR	6	TR
C18:1	0	0	0	0	0	0	0	2	4
C19:0	0	0	0	0	0	0	0	0	0
C20:0	0	0	0	0	0	0	0	TR	0

TR, Trace amount.

* Estimation of aldehyde size by comparison to retention time of saturated and mono-unsaturated standards.

† Most aldehyde products had in excess of 42 carbons and were not quantified.

Rhodococcus strain SL. The pyrolysis pattern of the *M. paratuberculosis* strain 116 differed from all of the other profiles as only one major methyl ester, C_{24}, eluted from the column (Fig. 2d).

16S rDNA sequence analysis

Three almost complete 16S rRNA sequences were obtained from oyster isolates NB4H (1473 nucleotides), RB1 (1513 nucleotides) and OB3P (1513 nucleotides). The three isolates had almost identical sequences (99·9–100·0 % similarity). The phylogenetic position of strain NB4H amongst the representative mycolic-acid-containing actinomycetes is shown in Fig. 3. This strain clearly belongs to the *N. otitidiscaviarum* sub-group and is most closely related to *N. nova* (97·6 % similarity), *N. otitidiscaviarum* (97·8 %) and *N. seriolae* (97·3 %), though these relationships are not supported by particularly high bootstrap values.

DISCUSSION

The biochemical, chemical, morphological and physiological characteristics of the seven bacterial isolates from diseased Pacific oysters from different locations were essentially identical. All of the isolates were Gram-positive, acid-fast, produced a mycelium which fragmented into irregular rod-like elements, had a peptidoglycan containing *meso*-DAP, arabinose and galactose as major sugars, mycolic acids with 46–58 carbon atoms and G + C-rich DNA. In addition, strain NB4H contained predominant amounts of hexahydrogenated menaquinones in which the two end units were cyclized. All of these properties are consistent with the classification of the isolates in the genus *Nocardia* (9, 11, 21). This conclusion is supported by the 16S rDNA sequence data. It is also interesting that the nucleotide similarities found between the test strains and the representatives of the validly described species of *Nocardia* are in the same order of magnitude as those found between representatives of the various established species (2).

It has been recommended that nomenclature should reflect genomic relationships (42), though confirmatory phenotypic data should be sought before new names are introduced (34). It is, therefore, particularly encouraging that the strains isolated from diseased Pacific oysters can be separated from representative nocardiae using a combination of biochemical, chemical and physiological properties. It was particularly

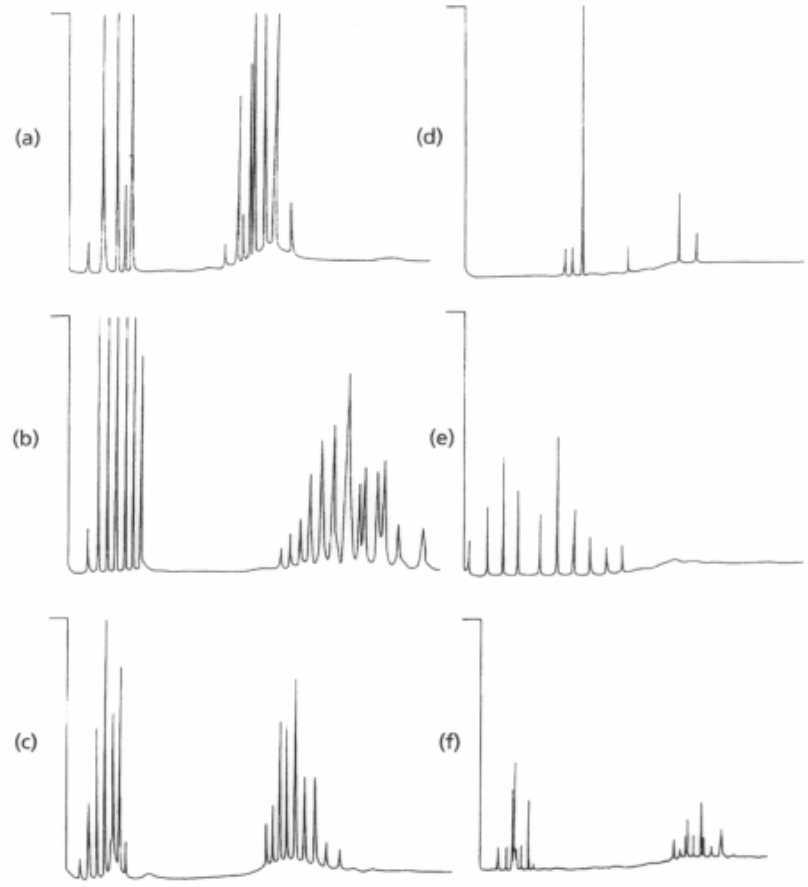

Fig. 2. Py-GLC profiles of methyl mycolates prepared from (a) *N. asteroides* strain GUH-2; (b) *N. seriolae* strain N-1; (c) strain RB29, isolated from a diseased Pacific oyster; (d) *M. paratuberculosis* strain 116; (e) *Rhodococcus* strain SL; and (f) the type strain NB4H, isolated from a diseased Pacific oyster.

interesting that the relative proportions and sizes of the aldehyde and methyl ester pyrolysis products differed amongst the test and reference strains (Fig. 2). The strains isolated from the aquatic hosts were characterized by the presence of more methyl esters with odd numbers of carbons than the other organisms studied. Similar results were reported by Kudo *et al.* (21) who compared *N. seriolae* (formerly '*Nocardia kampachi*'; 23, 24) and '*Nocardia salmonicida*' (a fish pathogen) with nocardiae isolated from terrestrial sources.

It is clear given the unique combination of genomic and phenotypic properties that the strains isolated from diseased Pacific oysters merit species status in the genus *Nocardia* (11). It is, therefore, proposed that the strains from the infected oysters be assigned to the genus *Nocardia* as *Nocardia crassostreae*.

Description of *Nocardia crassostreae* Friedman, Beaman, Chun, Goodfellow, Gee and Hedrick sp. nov.

Nocardia crassostreae (crass.os'tre.ae. M.L. n. *Crassostrea* generic name of Pacific oyster; M.L. gen. n. *crassostreae* of *Crassostrea*, referring to the source of the organism).

Aerobic, Gram-positive, acid-fast, non-motile actinomycetes which form a branched substrate mycelium which fragments into irregular rod-shaped forms. Pale yellow, waxy, wrinkled colonies are formed on BHI agar. Aerial hyphae are not formed. Strains have an oxidative metabolism and are catalase- and urease-positive but oxidase-negative. Degrades aesculin, Tweens 20 and 80, but not casein, gelatin, hypoxanthine, tyrosine or xanthine. Acid is produced from glucose, sucrose and trehalose, but not from D-arabinose, L-arabinose, galactose, maltose, D-mannitol, rhamnose or xylose. Gluconic acid, propionic acid, salicin, creatine and ethanol are not used as sole carbon sources. L-Valine is used as a sole carbon and nitrogen source but not L-arginine, lysine or ornithine. Grows at 22–30 °C and in the presence of isoniazid (200 μg ml^{-1}), lyzozyme, sodium chloride (2%, w/v) but not at 5 °C, at pH 9, or in the presence of 5-fluorouracil (20 μg ml^{-1}), mitomycin C (5 μg ml^{-1}), rifampicin (20 μg ml^{-1}) or sodium chloride (5%, w/v). The peptidoglycan contains *meso*-DAP and whole-organism hydrolysates have large amounts of arabinose and galactose. The organism contains [MK-8(H_4)C] menaquinones. Cell wall mycolic acids have 46–58 carbon atoms; on Py-GLC, large amounts of $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ methyl mycolates are released. The DNA G+C content is 68·6–69·3 mol%. Isolated from cultured Pacific oysters (*Crassostrea gigas*). Type strain: *Nocardia crassostreae* strain NB4H (= ATCC 700418).

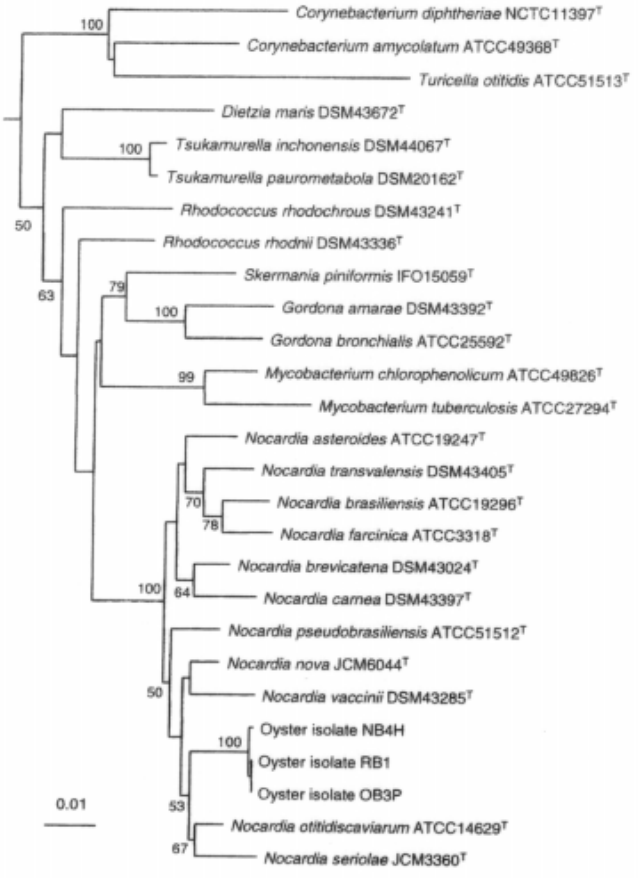

Fig. 3. Phylogenetic tree, constructed by neighbour-joining (37), showing the relationship between NB4H^T and strains RB1 and OB3P, isolated from a diseased Pacific oyster, and representatives of other mycolic-acid-containing taxa. The numbers at the nodes indicate the levels of bootstrap support on data for 1000 replicates; only values with $\geqslant 50\%$ significance are presented. The scale bar represents 0·01 substitutions per nucleotide position.

Further taxonomic studies are required to determine the relationship between *N. crassostreae* and what would seem to be a similar organism isolated from a diseased giant gourami, *Osphronemus goramy* (19). Such studies should provide insight into the host specificity and potential pathogenicity of nocardiae that infect aquatic hosts.

ACKNOWLEDGEMENTS

We thank Jeanette Weber, Mary Palieschekey, Don Alcendor, Douglas Nelson, Patti Brennan, James Clegg, Suzi Jackson, Robert Munn, Paul Lee and Robert Liddell for technical assistance. This research was funded, in part by the NOAA, National Sea Grant College Program, Department of Commerce (grant no. NA89AA-D-SG138), the California Sea Grant College Program (project no. R/A-74), and the Washington Sea Grant College Program (project no. R/A-73). This work was also funded by M. J. Murdock Charitable Trust, Vancouver, and Washington and Public Health Service research grant 1RO1-AI20900 from the National Institute of Allergy and Infections Diseases to B. L. Beaman. The authors are also indebted to the RDP for access to aligned sequence data. Jongsik Chun is grateful to the British Council (Seoul, Republic of Korea) for financial support.

REFERENCES

1. **Beaman, B. L. & Moring, S. E. (1988).** Relationship among cell wall composition, stage of growth and virulence of *Nocardia asteroides* GUH-2. *Infect Immun* **56**, 557–563.
2. **Chun, J. & Goodfellow, M. (1995).** A phylogenetic analysis of the genus *Nocardia* using 16S ribosomal RNA gene sequences. *Int J Syst Bacteriol* **45**, 240–244.
3. **Chun, J., Blackall, L. L., Kang, S.-O., Hah, Y. C. & Goodfellow, M. (1997).** A proposal to reclassify *Nocardia pinensis* (Blackall et al.) as *Skermania piniformis* gen. nov., comb. nov. *Int J Syst Bacteriol* **47**, 127–131.
4. **Davenport, D. J. & Johnson, G. C. (1986).** Cutaneous nocardiosis in a cat. *J Am Vet Med Assoc* **188**, 728–729.
5. **Felsenstein, J. (1985).** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
6. **Felsenstein, J. (1993).** PHYLIP (Phylogenetic Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle, USA.
7. **Friedman, C. S. & Hedrick, R. P. (1991).** Pacific oyster nocardiosis: isolation of the bacterium and induction of laboratory infections. *J Invertebr Pathol* **57**, 109–120.
8. **Friedman, C. S., Beattie, J. H., Elston, R. A. & Hedrick, R. P. (1991).** Investigation of the relationship between the presence of a Gram-positive bacterial infection and summer mortality of the Pacific oyster, *Crassostrea gigas* Thunberg. *Aquaculture* **94**, 1–15.
9. **Goodfellow, M. (1971).** Numerical taxonomy of some nocardioform bacteria. *J Gen Microbiol* **69**, 33–80.
10. **Goodfellow, M. (1992).** The family *Nocardiaceae*. In *The Prokaryotes*, 2nd edn, pp. 1188–1213. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
11. **Goodfellow, M. & Lechevalier, M. P. (1989).** Genus *Nocardia*. Trevisan 1889, 9^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1458–1471. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
12. **Goodfellow, M., Chun, J., Stubbs, S. & Teboli, A. S. (1994).** Transfer of *Nocardia amarae* Lechevalier and Lechevalier (1974) to the genus *Gordona* as *Gordona amarae* comb. nov. *Lett Appl Microbiol* **19**, 401–405.
13. **Gordon, R. E. (1966).** Some criteria for the recognition of *Nocardia madurae* (Vincent) Blanchard. *J Gen Microbiol* **45**, 355–364.
14. **Gordon, R. E. & Mihm, J. M. (1957).** A comparative study of some strains received as nocardiae. *J Bacteriol* **73**, 15–27.
15. **Gordon, R. E. & Mihm, J. M. (1962).** The type species of the genus *Nocardia*. *J Gen Microbiol* **27**, 1–10.
16. **Hoare, D. S. & Work, E. (1957).** The stereoisomers of IM-diaminopimelic acid. 2. Their distribution in the bacterial order *Actinomycetales* and in certain *Eubacteriales*. *Biochem J* **65**, 441–447.
17. **Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
18. **Kariya, T., Kubota, S., Nakamura, Y. & Kira, K. (1968).**

Nocardioform infection in cultured yellowtail (*Seriola quinqueradiata* and *S. purpurascens*). Bacteriological study. *Fish Pathol* **3**, 16–23 (in Japanese).

19. **Kitao, T., Ruangpan, L. & Fukudome, M. (1989).** Isolation and classification of a *Nocardia* species from diseased giant gourami, *Osphronemus goramy*. *J Aquat Anim Health* **1**, 154–162.

20. **Klatte, S., Rainey, F. A. & Kroppenstedt, R. M. (1994).** Transfer of *Rhodococcus aichiensis* Tsukamurella 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov. *Int J Syst Bacteriol* **44**, 769–773.

21. **Kudo, T., Hatai, K. & Seino, A. (1988).** *Nocardia seriolae* sp. nov. causing nocardiosis of cultured fish. *Int J Syst Bacteriol* **38**, 173–178.

22. **Kurup, P. V. & Schmitt, J. A. (1973).** Numerical taxonomy of *Nocardia*. *Can J Microbiol* **19**, 1035–1048.

23. **Kusuda, R. & Taki, H. (1973).** Studies on nocardial infection of cultured yellowtail. I. Morphological and biochemical characteristics of *Nocardia* isolated from diseased fishes. *Bull Japan Soc Fish* **39**, 937–943 (in Japanese).

24. **Kusuda, R., Taki, H. & Takeuchi, T. (1974).** Studies on nocardial infection of cultured yellowtail. II. Characteristics of *Nocardia kampachi* isolated from a gill-tuberculosis of yellowtail. *Bull Japan Soc Fish* **40**, 369–373 (in Japanese).

25. **Larsen, W., Olsen, G. J., Maidak, B. L., McCaughey, M. J., Overbeek, R., Macke, T. J., Marsh, T. L. & Woese, C. R. (1993).** The ribosomal database project. *Nucleic Acids Res* **21**, 3021–3023.

26. **Lechevalier, M. P. & Lechevalier, H. A. (1970).** A critical evaluation of the genera of aerobic actinomycetes. In *The Actinomycetales*, pp. 393–405. Edited by H. Prauser. Jena: Gustav Fischer Verlag.

27. **Lechevalier, M. P., Horan, A. C. & Lechevalier, H. (1971).** Lipid composition in the classification of nocardiae and mycobacteria. *J Bacteriol* **105**, 313–318.

28. **Lechevalier, M. P., Lechevalier, H. & Horan, A. C. (1973).** Chemical characteristics and classification of nocardiae. *Can J Microbiol* **19**, 965–972.

29. **McNeil, M. M. & Brown, J. M. (1994).** The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin Microbiol Rev* **7**, 357–417.

30. **Minnikin, D. E., Hutchison, I. G., Caldicott, A. B. & Goodfellow, M. (1980).** Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr* **188**, 221–223.

31. **Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Pariett, J. H. (1984).** An integrated procedure for the extraction of isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.

32. **Murray, R. G. E. & Proctor, A. G. J. (1965).** Paper chromatography as an aid to the identification of *Nocardia* species. *J Gen Microbiol* **41**, 163–167.

33. **Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N. P., Stackebrandt, E. & Zavarzin, G. A. (1990).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on approaches to taxonomy within the *Proteobacteria*. *Int J Syst Bacteriol* **40**, 213–215.

34. **Nelson, D. C., Waterbury, J. B. & Jannasch, H. W. (1984).** DNA base composition and genome size of the prokaryotic symbiont in *Riftia pachyptila* (Pogonophora). *FEMS Microbiol Lett* **24**, 267–271.

35. **Ruimy, R., Boiron, P., Boivin, V. & Christen, R. (1994).** A phylogeny of the genus *Nocardia* deduced from the analysis of small-subunit ribosomal DNA sequences, including transfer of *Nocardia amarae* to the genus *Gordona* as *Gordona amarae* comb. nov. *FEMS Microbiol Lett* **123**, 261–268.

36. **Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

37. **Sierra, G. (1957).** A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie Leeuwenhoek* **23**, 15–22.

38. **Snieszko, S. F., Bullock, G. L., Dunbar, C. E. & Pettijohn, L. L. (1964).** Nocardial infection in hatchery-reared fingerling rainbow trout (*Salmo gairdneri*). *J Bacteriol* **88**, 1809–1810.

39. **Staneck, J. L. & Roberts, G. D. (1974).** Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.

40. **Steadham, J. E. (1979).** Reliable urease test identification of mycobacteria. *J Clin Microbiol* **10**, 134–137.

41. **Thole, J. E. R., Dauwerse, H. G., Das, P. K., Groothius, D. G., Schouls, L. M. & Van Embden, J. D. A. (1985).** Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. *Infect Immun* **50**, 800–806.

42. **Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

43. **Whipple, D. L., LeFebre, R. B., Andrews, R. E., Jr & Thiermen, A. B. (1987).** Isolation and analysis of restriction endonuclease digestive patterns of chromosomal DNA from *Mycobacterium paratuberculosis* and other *Mycobacterium* species. *J Clin Microbiol* **25**, 1511–1515.