***Nocardia salmonicida* nom. rev., a fish pathogen**

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An almost complete gene sequence of 16S rDNA of '*Nocardia salmonicida*' strain JCM 4826^T was determined following cloning and sequencing of the amplified gene. The sequence was aligned with those available for nocardiae and phylogenetic trees inferred using four tree-making algorithms. The organism and the type strain of *Nocardia asteroides* consistently formed a monophyletic clade with a distant sequence similarity of 97%. However, previous DNA relatedness experiments showed that strain JCM 4826^T and *Nocardia asteroides* ATCC 19247^T belong to different genomic species. The organism was also distinguished from representatives of all validly described species of *Nocardia* using a combination of phenotypic features. The polyphasic evidence showed that the strain merits recognition as a new species of the genus *Nocardia*. The name proposed for the new species is *Nocardia salmonicida* nom. rev.

Keywords: *Nocardia salmonicida* nom. rev., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

The application of modern taxonomic methods promoted a radical reappraisal of the genus *Nocardia* (Trevisan, 1889; Goodfellow, 1997). The revised genus encompasses 13 validly described species which form a distinct monophyletic clade within the evolutionary radiation occupied by mycolic acid-containing actinomycetes, that is the mycolata (Chun & Goodfellow, 1995; Chun *et al.*, 1997, 1998; Friedman *et al.*, 1998). The improved classification provides a sound framework for the recognition of additional species of *Nocardia*, including taxa which currently do not have any official standing in bacterial nomenclature.

Strain JCM 4826^T, a fish pathogen isolated from blueblack salmon (*Oncorhynchus nerka*), was assigned to the genus *Streptomyces* as *Streptomyces salmonicida* (Rucker, 1949) but was transferred to the genus *Nocardia* as *Nocardia salmonicida* when whole-organism hydrolysates of the strain were found to contain meso-diaminopimelic acid, arabinose and galactose (Pridham & Lyons, 1969; Pridham, 1970). This reclassification was subsequently underpinned by additional chemotaxonomic and morphological data (Goodfellow, 1971; Alderson *et al.*, 1985; Kudo *et al.*,

1988) and the organism was shown to be most closely related to *Nocardia asteroides* on the basis of the results from DNA:DNA relatedness (Mordarski *et al.*, 1977; Kudo *et al.*, 1988) and numerical phenetic (Orchard & Goodfellow, 1980) studies. '*Nocardia salmonicida*' JCM 4826^T also fell outwith the *Streptomyces* clusters in the numerical phenetic survey of Williams *et al.* (1983). The taxon was neither cited in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) nor recognized in the current edition of *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989).

The aim of the present investigation was to clarify the relationships of '*Nocardia salmonicida*' JCM 4826^T using a polyphasic taxonomic approach. It was evident from the resultant genotypic and phenotypic data that the organism should be recognized as a new species of *Nocardia* for which the name *Nocardia salmonicida* nom. rev. is proposed.

METHODS

Bacterial strains. '*Nocardia salmonicida*' JCM 4826^T (= NRRL B-2778^T) was grown in shake flasks containing modified Sauton's broth (Mordarska *et al.*, 1972) for 7 d at 30 °C; biomass was harvested by centrifugation and washed twice with distilled water. Additional strains were examined in the phenotypic tests, namely *Nocardia pseudobrasilensis* N1237^T (P. Boiron, Institut Pasteur, Paris; CIP 104600^T),

The GenBank accession number for the 16S rDNA sequence of strain JCM 4826^T is Z46750.

N1234 (P. Boiron; N249), N1235 (P. Boiron; N649) and N1236 (P. Boiron; N51511); *Nocardia seriolae* N1116^T (JCM 3359), N1115 (JCM 3360), N1118 (JCM 5849) and N1119 (JCM 5850); and *Nocardia transvalensis* N1202^T (DSM 43405^T), N1213 (N. Poonwan, Department of Medical Sciences, Northaburi, Thailand; 35-157-10), N1214 (N. Poonwan; 34-104-03) and N1215 (N. Poonwan; 34-43-6). All of the strains were maintained as glycerol suspensions (20 %, v/v) at -20 °C.

Phenotypic characterization. The colonial properties of the test strains were determined on glucose yeast extract agar (GYEA; Gordon & Mihm, 1962) plates after 14 d incubation at 30 °C. Smears from these plates were prepared, Gram-stained after Hucker's modification (Society for American Bacteriologists, 1957) and examined for micro-morphological features. Smears were also stained using a modification of the Ziehl-Neelson method (Gordon, 1967) and the degree of acid-fastness noted. Unless otherwise stated, the remaining tests were read after incubation for 14 d at 30 °C. The degradation of adenine (0.4 %), elastin (0.3 %), hypoxanthine (0.4 %), testosterone (0.1 %), L-tyrosine (0.5 %) and xanthine (0.4 %) were determined using GYEA as the basal medium with incubation for 21 d (Goodfellow, 1971). The degradation of uric acid was detected using nutrient agar as the basal medium (Boiron *et al.*, 1993); zones of clearing around colonies were scored as positive after incubation for 21 d. Established procedures were used to detect aesculin hydrolysis (Williams *et al.*, 1983), nitrate reduction (Gordon & Mihm, 1962) and urea hydrolysis (Rustigan & Stuart, 1941). The ability of the strains to grow on carbon compounds (Table 1) as sole sources of carbon for energy and growth was examined using Stevenson's basal medium (Stevenson, 1967). Growth at 45 °C was recorded on GYEA plates.

16S rDNA sequencing. Isolation of chromosomal DNA, and PCR, cloning and sequencing of the 16S rDNA of strain JCM 4826^T were carried out using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit and a 373A DNA Sequencer (Applied Biosystems) as described previously (Chun & Goodfellow, 1995). The resultant 16S rDNA sequence was aligned manually against sequences of representative mycolata strains; the additional sequence data were retrieved from the GenBank and EMBL databases. Phylogenetic trees were inferred using the Fitch-Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) treeing algorithms. Evolutionary distance matrices for the neighbour-joining and Fitch-Margoliash methods were generated according to Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all the analyses. The resultant unrooted tree topologies were evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

Chemotaxonomy. Freeze-dried biomass of strain JCM 4826^T (50 mg) was degraded by acid methanolysis and hexane extracts examined for mycolic acids by TLC as described by Minnikin *et al.* (1975, 1980). Menaquinone was analysed using HPLC as described previously (Chun & Goodfellow, 1995).

RESULTS AND DISCUSSION

An almost complete 16S rDNA sequence (1471 nt) was obtained for strain JCM 4826^T. Comparison of this sequence with corresponding nucleotide sequences of

representative actinomycetes showed that the organism belongs to the genus *Nocardia* (data not shown). This result confirms and extends data from previous studies which indicated that strain JCM 4826^T has morphological and chemotaxonomic properties consistent with its assignment to the genus *Nocardia* (Pridham & Lyons, 1969; Orchard & Goodfellow, 1980; Alderson *et al.*, 1985; Kudo *et al.*, 1988). It was also shown that strain JCM 4826^T contains mycolic acids with an R_F value on TLC plates within the range typical of nocardiae. In addition, the strain contained predominant amounts of hexahydrogenated menaquinones with eight isoprene units where the end two units were cyclized; this menaquinone profile has been found only in members of the genera *Nocardia* and *Skermania* (Chun & Goodfellow, 1995; Chun *et al.*, 1997).

The unrooted evolutionary tree (Fig. 1) shows that strain JCM 4826^T forms a monophyletic clade with *Nocardia asteroides*. This relationship was highlighted in the analyses based on all four treeing algorithms by the relatively high nucleotide similarity value (97 %) and the high bootstrap value (76 %) based on the neighbour-joining method. This relationship between '*Nocardia salmonicida*' JCM 4826^T and *Nocardia asteroides* strains, including the type strain, is also supported by DNA-DNA relatedness studies (Mordarski *et al.*, 1977; Kudo *et al.*, 1988) where the relatedness values between the two strains ranged from 45 to 57 %. However, it is apparent from these studies that strain JCM 4826^T forms a distinct genomic species within the genus *Nocardia*. It is also clear from both the present and earlier studies that strain JCM 4826^T can be distinguished from representatives of all of the validly described species of *Nocardia*, including *Nocardia asteroides*, using a battery of phenotypic properties (Table 1).

The genotypic and phenotypic data indicate that strain JCM 4826^T merits recognition as a distinct species in the genus *Nocardia*. It is therefore proposed that the organism be classified in the genus *Nocardia* as *Nocardia salmonicida* nom. rev.

Description of *Nocardia salmonicida* nom. rev.

Nocardia salmonicida (sal.mo.ni'ci.da. L. n. *salmo*, *salmonis* salmon; L. suff. *cida* from L. v. *caedo* to cut or kill; M.L. n. *salmonicida* salmon-killer).

The description is based on data taken from this and previous studies (Rucker, 1949; Shirling & Gottlieb, 1972; Orchard & Goodfellow, 1980; Kudo *et al.*, 1988). Aerobic, Gram-positive, catalase-positive, partially acid-alcohol-fast, non-motile actinomycete which produces an extensively branched substrate mycelium which fragments *in situ* into rod-shaped to coccoid elements. An orange substrate mycelium carries white to pink aerial hyphae. Colony elevation is convex to irregular and colony margins are filamentous. Diffusible pigments are not formed. Tes-

Fig. 1. Unrooted neighbour-joining tree based on 1407 unambiguously aligned nucleotide positions. Asterisks indicate the branches that were also recovered using the Fitch–Margoliash, maximum-likelihood and maximum-parsimony methods. The numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. The scale represents 0.01 nt substitutions per position.

Table 1. Phenotypic characters which distinguish strain JCM 4826^T from other nocardiae

Data taken from this study and from Orchard & Goodfellow (1980), Goodfellow (1997), Chun *et al.* (1998) and Friedman *et al.* (1998). +, >90 % of strains positive; –, >90 % of strains negative; ND, not determined.

Character	JCM 4826 ^T	<i>N. asteroides</i>	<i>N. brasiliensis</i>	<i>N. brevicatena</i> DSM 43024 ^T	<i>N. carnea</i>	<i>N. crassostreae</i>	<i>N. farcinica</i>	<i>N. flavorosea</i>	<i>N. nova</i>	<i>N. otitidiscaviarum</i>	<i>N. pseudobrasiliensis</i>	<i>N. seriolae</i>	<i>N. transvalensis</i>	<i>N. vaccinii</i> DSM 43285 ^T
Biochemical tests														
Aesculin hydrolysis	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Nitrate reductase	+	+	+	–	+	ND	+	–	+	+	–	–	+	+
Urea hydrolysis	+	+	+	–	–	–	+	–	+	+	+	–	+	+
Decomposition of:														
Adenine	–	–	–	–	–	ND	–	–	–	–	+	–	–	–
Casein	–	–	+	–	–	–	–	–	–	–	+	–	–	–
Elastin	–	–	+	–	–	ND	–	–	–	–	+	–	+	–
Hypoxanthine	–	–	+	–	–	–	–	–	–	+	+	–	+	–
Testosterone	+	+	+	+	+	ND	+	+	+	–	–	–	+	–
Tyrosine	+	–	+	–	–	–	–	–	–	–	+	–	–	–
Uric acid	–	–	–	ND	–	ND	–	–	–	+	+	–	+	–
Xanthine	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Growth on sole carbon sources														
D(+)-Mannitol (1 %)	+	–	+	–	+	ND	–	+	–	+	+	–	+	+
α-L(–)-Rhamnose (1 %)	–	–	–	+	–	ND	+	–	–	–	–	–	+	+
D(+)-Sorbitol (1 %)	+	–	–	–	+	ND	–	+	–	–	+	–	+	–
Citric acid (sodium salt) (0.1 %)	+	+	+	–	–	–	–	+	–	–	+	+	–	–
Growth at 45 °C	–	–	+	–	–	–	+	–	–	+	–	–	–	–

tosterone, L-tyrosine and Tweens 20 and 80 are degraded, but not adenine, casein, cellulose, chitin, elastin, guanine, hypoxanthine, starch, xanthine or xylan. Nitrate is reduced and aesculin and urea hydrolysed. Acid is formed from D(+)-glucose and glycerol but not from L(–)-arabinose, *meso*-erythritol, D(+)-galactose, D(+)-maltose, D(+)-mannose, α-L-

rhamnose or D(+)-trehalose. D(–)-Fructose, D(+)-glucose, D(+)-mannitol, D(+)-sorbitol, butyrate, citrate, fumarate, malate, propionate and succinate are used as sole carbon sources for energy and growth but not amygdalin, D(–)- or L(–)-arabinose, arbutin, D(+)-cellobiose, dulcitol, D(+)-galactose, glycogen, *meso*-inositol, inulin, D(+)-melezitose, D(+)-

raffinose, α -L-rhamnose, acetamide, acetate, anthranilic acid, benzoate, 1,4-butanediol, 2,3-butanediol, hippurate, 4-hydroxybenzoate, lactate, malonate, 2-octanol, pimelic acid or tartrate. Acetamide, L-cysteine, L-glutamate, L-serine, sodium nitrate, $(\text{NH}_4)_2\text{HPO}_4$, pyrazinamide, L-serine and urea are used as sole nitrogen sources but not L-phenylalanine. Neither monoethanolamine nor trimethylenediamine are used as sole sources of carbon and nitrogen. Growth occurs between 20 and 30 °C and in the presence of bismuth citrate (0.0001%), crystal violet (0.00001%), phenol (0.01%), sodium chloride (3%, w/v) and lysozyme but not at 10 or 35 °C, nor in the presence of sodium azide (0.01%) or sodium chloride (10%, w/v). The major cellular fatty acids are hexadecanoic (32% of total fatty acids), hexadecenoic (14%), octadecenoic (16%) and 10-methyloctadecenoic (25%) acids. The G+C ratio of the DNA is 67 mol%. The organism is pathogenic for cultured fish. The type strain is JCM 4826^T (= ATCC 27463^T = CBS 694.72^T = IFO 13393^T = ISP 5472^T = NRRL B-2778^T). The species description is based on a single strain and hence serves as the type strain description.

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