Gordonia defluvii sp. nov., an actinomycete isolated from activated sludge foam

Jacques A. Soddell,¹ Fiona M. Stainsby,^{2†} Kathryn L. Eales,¹
Robert J. Seviour¹ and Michael Goodfellow²

Correspondence

Michael Goodfellow
m.goodfellow@ncl.ac.uk

¹Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3552, Australia

²Division of Biology, University of Newcastle, Newcastle upon Tyne NE1 7RU, UK

Three strains of non-motile, Gram-positive, filamentous actinomycetes, isolates J4^T, J5 and J59, initially recognized microscopically in activated sludge foam by their distinctive branching patterns, were isolated by micromanipulation. The taxonomic positions of the isolates were determined using a polyphasic approach. Almost-complete 16S rRNA gene sequences of the isolates were aligned with corresponding sequences of representatives of the suborder *Corynebacterineae* and phylogenetic trees were inferred using three tree-making algorithms. The organisms formed a distinct phyletic line in the *Gordonia* 16S rRNA gene tree. The three isolates showed 16S rRNA gene sequence similarities within the range 96.9–97.2% with their nearest phylogenetic neighbours, namely *Gordonia bronchialis* DSM 43247^T and *Gordonia terrae* DSM 43249^T. Strain J4^T was shown to have a chemotaxonomic profile typical of the genus *Gordonia* and was readily distinguished from representatives of the genus on the basis of Curie-point pyrolysis mass spectrometric data. The isolates shared nearly identical phenotypic profiles that distinguished them from representatives of the most closely related *Gordonia* species. It is evident from the genotypic and phenotypic data that the three isolates belong to a novel *Gordonia* species. The name proposed for this taxon is *Gordonia defluvii* sp. nov.; the type strain is J4^T (=DSM 44981^T=NCIMB 14149^T).

Most activated sludge systems suffer intermittently from the serious operational disorder known as foaming or scumming, whereby a stable foam or scum develops on the surface of aeration tanks (Soddell, 1999). The reasons for the appearance of such foams are not clear, but their stable nature is imparted by the presence of large numbers of bacteria in the foam layer. In many cases, these foam-associated bacteria are mycolic acid-containing actinomycetes that belong to the suborder *Corynebacterineae* Stackebrandt *et al.* 1997. It is generally accepted that mycolic acids render cells sufficiently hydrophobic to allow them to accumulate on the surfaces of aeration tanks (Seviour & Blackall, 1999).

Initially, *Nocardia amarae* (now *Gordonia amarae* Klatte *et al.* 1994) was implicated in foaming (Lechevalier & Lechevalier, 1974), but it is apparent from culture-dependent and -independent approaches that diverse populations of mycolic acid-containing actinomycetes are involved. Isolates

representing the genera *Millisia* (Soddell *et al.*, 2006) and *Skermania* (Blackall *et al.*, 1989; Chun *et al.*, 1997) have been recovered only from activated sludge foams. Novel species isolated from foams include members of the genera *Nocardia* (Yamamura *et al.*, 2005) and *Tsukamurella* (Nam *et al.*, 2003, 2004). However, it is clear that many additional mycolic acid-containing taxa associated with foam need to be formally described (Soddell & Seviour, 1998; Stainsby *et al.*, 2002). Such studies are important, as attempts to control the appearance and persistence of foams are unlikely to succeed until the taxonomic diversity and functional roles of the causal organisms are understood (Goodfellow *et al.*, 1996, 1998; Stainsby *et al.*, 2002).

Three isolates with a cellular morphology intermediate between the right-angled branching pattern typical of *G. amarae* Klatte *et al.* 1994 and the 'pine-tree-like' morphology of *Skermania piniformis* Chun *et al.* 1997 were isolated, by micromanipulation, in Australia from activated sludge foams at Brimbank Park, Victoria (isolates J4^T and J5) and Craigieburn, Victoria (isolate J59), as described by Soddell & Seviour (1998). These authors considered that the strains might form a novel species on the basis of an extensive numerical taxonomic survey of *Skermania* and related strains, a proposition underpinned by the results of the present investigation.

[†]Present address: School of Life Sciences, Napier University, Edinburgh EH10 5DT, UK.

Abbreviation: PyMS, Curie-point pyrolysis mass spectrometry.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains J4^T, J5 and J59 are AY650265–AY650267, respectively.

Extraction of chromosomal DNA and PCR amplification and sequencing of 16S rRNA genes of the three isolates were carried out as described previously (Soddell *et al.*, 2006). The resultant 16S rRNA gene sequence data were compared with corresponding results taken from the RDP database for representatives of genera classified in the sub-order *Corynebacterineae*. Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the Jukes & Cantor (1969) distance model. The unrooted tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset using the SEQBOOT and CONSENSE options from the PHYLIP package (Felsenstein, 1989).

Almost full-length 16S rRNA gene sequences (1519 nucleotides) that corresponded to *Escherichia coli* positions 27–1525 were obtained for the three isolates. A comparison of these sequences with those of representatives of the sub-order *Corynebacterineae* showed that the isolates fell within the evolutionary radiation occupied by the genus *Gordonia* (data not shown). It is evident from Fig. 1 that the isolates form a distinct lineage within the *Gordonia* 16S rRNA gene tree, a phyletic line that is supported by all of the tree-making algorithms and by a 100 % bootstrap value. The

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing that isolates J4^T, J5 and J59 form a distinct phyletic line in the *Gordonia* 16S rRNA gene tree. Asterisks indicate branches that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. Bar, 0.01 substitutions per nucleotide position. The outgroup strain used to root the tree (not shown) was *Turicella otitidis* 234/92^T (GenBank accession no. X73976).

isolates shared 16S rRNA gene sequence similarities within the range 99.7–99.9 %, values equivalent to between 2 and 5 nucleotide differences; all but two of the latter were in conserved regions.

It is also apparent from Fig. 1 that the isolates are loosely associated with the type strains of *G. amarae* (96.2–96.4 % similarity) and *Gordonia hirsuta* (96.0–96.2 % similarity), though these relationships are not supported by a high bootstrap value. Indeed, the isolates are not closely related to any of the *Gordonia* type strains; their closest relatives are *Gordonia bronchialis* DSM 43247^T (96.9–97.2 % similarity) and *Gordonia terrae* DSM 43249^T (96.7–97.1 % similarity). Given the low values, it was not necessary to carry out DNA–DNA pairing experiments between isolate J4^T and its closest phylogenetic neighbours, especially since similarity values well above these levels have been reported between representatives of several species of *Gordonia* with validly published names. The type strains of *Gordonia alkanivorans* and *Gordonia rubripertincta*, for example, share 99.1 % 16S rRNA gene sequence similarity and have a DNA–DNA relatedness of 52 % (Kummer *et al.*, 1999), a value well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the recognition of genomic species.

Strain J4^T was the subject of chemotaxonomic studies designed to determine whether it had chemical markers consistent with its classification in the genus *Gordonia*. Standard methods were used for the extraction and analysis of fatty acids (Midi system; <http://www.midi-inc.com>), isoprenoid quinones (Minnikin *et al.*, 1984), muramic acid type (Uchida *et al.*, 1999), mycolic acids (Minnikin *et al.*, 1980), polar lipids (Minnikin *et al.*, 1984) and sugars (Schaal, 1985). The isolate contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), *N*-glycolated muramic acid, dihydrogenated menaquinones with nine isoprene units [MK-9(H₂)] as the predominant isoprenologue with small amounts of MK-8(H₂) and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol dimannosides, phosphatidylethanolamine and an unidentified glycolipid as major polar lipids (phospholipid type 2 *sensu* Lechevalier *et al.*, 1977). It also contained major proportions of oleic (C_{18:1}; 35.2 % of the total fatty acid composition), palmitic (C_{16:0}; 21.3 %), palmitoleic (C_{16:1}; 17.5 %) and tuberculostearic (9.0 %) acids and minor proportions of myristic (3.7 %), margaric (2.7 %), eicosenoic (2.7 %), stearic (2.4 %), heptadecanoic (2.2 %), nonadecenoic (2.1 %) and pentadecylic (1.2 %) acids. The organism possessed mycolic acids, the methyl esters of which had the same R_f value after thin-layer chromatography as those of *G. bronchialis* DSM 43247^T. All of these properties are in line with the assignment of the strain to the genus *Gordonia* (Goodfellow & Maldonado, 2006).

The DNA G+C content was determined for strain J4^T; isolation and purification of the DNA was carried out after Pitcher *et al.* (1989). The base composition of the resultant DNA preparation was determined using the reversed-phase

Fig. 2. Three-dimensional ordination diagram generated from PyMS data showing relationships between isolate J4^T and the type strains of representative *Gordonia* species. The plot is based on the first three canonical variates of a principal component-canonical variates analysis that accounted for 93% of the total variation found between the tested strains.

HPLC procedure described by Tamaoka (1994). The molar G+C ratio calculated after Mesbah *et al.* (1989) was 63.1 mol%.

Curie-point pyrolysis mass spectrometry (PyMS) has been used to good effect to distinguish between closely related organisms, including members of mycolic acid-containing taxa (Goodfellow *et al.*, 1997). A standard PyMS procedure (Goodfellow *et al.*, 1998) was used to determine relationships between isolate J4^T and representatives of the genus *Gordonia*. It is apparent from Fig. 2 that strain J4^T can be readily distinguished from the *Gordonia* marker strains, including the type strains of *G. amarae*, *G. bronchialis*, *G. hirsuta* and *G. terrae*. In addition, all three isolates can be distinguished from representatives of these and other phylogenetically close species using a combination of phenotypic properties (Table 1) and by their characteristic micromorphology.

It is clear from the genotypic and phenotypic data that isolates J4^T, J5 and J59 merit recognition within a novel species in the genus *Gordonia*. The name *Gordonia defluvii* sp. nov. is proposed for this taxon.

Description of *Gordonia defluvii* sp. nov.

Gordonia defluvii (de.flu'vi.i. L. gen. n. *defluvii* of sewage).

The description is taken from this and the earlier study of Soddell & Seviour (1998). Aerobic, Gram-positive, slightly

Table 1. Phenotypic characteristics that separate isolates J4^T, J5 and J59 from representatives of closely related *Gordonia* species

Strains: 1, isolates J4^T, J5 and J59; 2, *G. amarae* DSM 43392^T; 3, *G. bronchialis* DSM 43247^T; 4, *G. hirsuta* DSM 44140^T; 5, *G. hydrophobica* DSM 44015^T; 6, *G. rubripertincta* DSM 43197^T; 7, *G. terrae* DSM 43249^T. Data for *Gordonia* type strains were taken from Maldonado *et al.* (2003).

Characteristic	1	2	3	4	5	6	7
Colour of colony*	NP	T/W	B	W/LW	T/W	O/R	P/O
Biochemical tests							
Aesculin hydrolysis	—	+	—	+	+	—	+
Allantoin hydrolysis	—	+	—	—	+	—	—
Nitrate reductase	+	—	+	+	+	—	+
Urea hydrolysis	—	+	+	—	—	+	+
Degradation of: (% w/v)							
Hypoxanthine (0.4)	—	+	—	—	+	+	+
Starch (1)	—	+	+	+	—	+	+
Tributyrin (0.1)	+	—	—	—	—	+	—
Tween 80 (1)	+	—	—	+	+	—	—
Tyrosine (0.5)	—	+	—	—	+	—	—
Uric acid (0.5)	+	+	—	—	+	+	+
Xanthine (0.4)	—	—	—	—	—	—	+
Growth on sole carbon sources (% w/v)							
Arbutin (1)	+	+	—	+	+	—	—
D-Cellobiose (1)	—	—	—	+	—	+	—
Glycerol (1)	—	+	+	+	+	+	+
N-Acetylglucosamine (0.1)	+	+	—	+	+	—	—
Betaine (0.1)	+	—	—	—	—	—	+
Sodium fumarate (1)	—	—	+	—	+	+	+

*B, Brown; LY, light yellow; NP, non-pigmented; O, orange; P, pink; R, red; T, tan; W, white.

acid–alcohol-fast, non-motile, non-spore-forming, slowly growing actinomycete that shows acute-angled and right-angled branching. Non-pigmented colonies with filamentous margins and abundant aerial hyphae are formed on glucose-yeast extract agar. Diffusible pigments are not produced. The organism is catalase- and phosphatase-positive but does not produce *m*- or *p*-nitrophenol oxidases. Esterase lipase (C₈), leucine aminopeptidase and β -glucosidase are produced but not alkaline phosphatase, chymotrypsin, α -fucosidase, α - or β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, trypsin or valine aminopeptidase. Degrades Tween 20 but not adenine, casein or elastin. Pyruvate is used as a sole carbon source but not arabinose, fructose, galactose, maltose, mannose, melezitose, rhamnose, salicin or sorbitol (all at 0.1 %, w/v) or acetamide, benzamide, benzoate, butyrate, citrate, *p*-cresol, gluconate, *m*- or *p*-hydroxybenzoic acid, lactate, malate, octanoate, oxalate, pimelate, propionate, sebacate, succinate, tartrate, testosterone or tyrosine (all at 0.01 %, w/v). Grows from pH 5 to 8 and between 15 and 30 °C, albeit weakly at these latter temperatures. Does not grow in the presence of crystal violet (0.001 %), phenol (0.01 %, w/v), phenol ethanol (0.03 %, v/v), sodium azide (0.01 %, w/v), sodium chloride (5 %, w/v) or penicillin (10 IU). Additional phenotypic properties are shown in Table 1. Chemo-taxonomic properties are typical of the genus *Gordonia*. The G + C content of the genomic DNA is 63.1 mol%.

The type strain, J4^T (= DSM 44981^T = NCIMB 14149^T), was isolated from a foam sample taken from an activated sludge plant at Brimbank Park, Victoria, Australia.

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