***Millisia brevis*** gen. nov., sp. nov., an actinomycete isolated from activated sludge foam

Jacques A. Soddell,1 Fiona M. Stainsby,2 Kathryn L. Eales,1 Reiner M. Kroppenstedt,3 Robert J. Seviour1 and Michael Goodfellow2

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

2Division of Biology, King George Vith Building, University of Newcastle, Newcastle upon Tyne NE1 7RU, UK

3DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, 38124 Braunschweig, Germany

The taxonomic position of two mycolic-acid-producing actinomycetes, isolates J81T and J82, which were recovered from activated sludge foam, was clarified. Comparative 16S rRNA gene sequence studies indicated that the organisms formed a distinct lineage within the *Corynebacterineae* 16S rRNA gene tree. The taxonomic integrity of this group was underpinned by a wealth of phenotypic data, notably characteristic rudimentary right-angled branching. In addition, isolate J81T contained the following: meso-diaminopimelic acid, arabinose and galactose; *N*-glycocolated muramic acid residues; a dihydrogenated menaquinone with eight isoprene units as the predominant isoprenologue; a fatty acid profile rich in oleic and palmitoleic acids and with relatively small proportions of myristic, stearic and tuberculostearic acids; mycolic acids with 44–52 carbons; and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol mannosides as major polar lipids. Strain J81T was found to have a chemotaxonomic profile that serves to distinguish it from representatives of all of the other taxa classified as belonging to the suborder *Corynebacterineae*. In the light of these data, it is proposed that the two isolates be classified in a novel monospecific genus. The name proposed for this taxon is *Millisia brevis* gen. nov., sp. nov.; strain J81T (=DSM 44463T=NRRL B-24424T) is the type strain of *Millisia brevis*.

The taxonomy of mycolic-acid-containing actinomycetes has been clarified and extended by the application of genotypic and phenotypic procedures to representatives of established and novel taxa (Goodfellow et al., 1998a, 1999; Gürtler et al., 2004). Actinomycetes characterized by the presence of mycolic acids are classified in the suborder *Corynebacterineae* Stackebrandt et al. 1997, which encompasses the genera *Corynebacterium*, Dietzia, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Sanguiniparus*, *Skermania*, *Tsukamurella* and *Williamsia* (Butler et al., 2005; Goodfellow & Maldonado, 2006). Members of these taxa can be distinguished from one another using a combination of chemotaxonomic and morphological properties, and form distinct lineages in the *Corynebacterineae* 16S rRNA gene tree. Activated sludge wastewater-treatment plants with foaming problems (Soddell, 1999) are a rich source of mycolic-acid-containing actinomycetes (Lemmer & Kroppenstedt, 1984; Soddell & Seviour, 1990, 1995, 1998; Goodfellow et al., 1998b; Stainsby et al., 2002), as exemplified by *Gordonia amarae* (Lechevalier & Lechevalier, 1974) Klatte et al. 1994, *Skermania piniformis* Chun et al. 1997, *Tsukamurella pseudospumae* Nam et al. 2004 and *Tsukamurella spumae* Nam et al. 2003.

Two actinomycetes that showed rudimentary right-angled branching and which produced salmon-pink filamentous colonies were isolated from activated sludge foam by using a micromanipulator (Soddell & Seviour, 1994). Subsequent studies based on numerical taxonomic and preliminary 16S rRNA gene sequence data indicated that the two strains, isolates J81T and J82, might represent a novel genus in the suborder *Corynebacterineae* (Soddell & Seviour, 1998; Soddell et al., 1998), a proposition underpinned by the results of the present investigation.

DNA from strains J81T and J82 was extracted with a MoBio Laboratories UltraClean Soil DNA kit (Geneworks). 16S rRNA genes were PCR-amplified with universal primers 27f and 1525r, amplicons were purified with a QIA Quick PCR purification kit (Qiagen) and cloning was completed using pGEM-T Easy vector system II (Promega). The resultant preparations were fully sequenced with primers M13F,
M13R, 530F and 907R with Applied Biosystems PRISM BigDye 3.1 Terminator chemistry and then sequenced at the DNA Micromon Sequencing Facility of Monash University (Clayton, Australia). The 16S rRNA gene sequence results were compared with corresponding data retrieved from the Ribosomal Database Project (http://rdp.cme.msu.edu/) for representatives of the genera classified in the suborder Corynebacterineae. Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the distance model of Jukes & Cantor (1969). The unrooted tree topologies were subjected to bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset, using the SEQBOOT and CONSENSE options from the PHYLIP package (Felsenstein, 1989).

Almost-complete 16S rRNA gene sequences (1519 nt) corresponding to Escherichia coli positions 27–1525 were obtained for the two strains. It is evident from Fig. 1 that the organisms have identical 16S rRNA gene sequences and form a deep-rooted lineage in the Corynebacterineae tree that can be equated with branches corresponding to recognized genera, a result consistent with their recognition as members of a novel genus. The strains tested are most closely related to members of Gordonia species with validly published names (93.7–95.7% similarity) and to the type strain of S. piniformis (95.6% similarity).

Chemotaxonomic studies were carried out to determine whether J81T merited generic status within the suborder Corynebacterineae. Standard procedures 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 (Uchida et al., 1999), mycolic acids (Minnikin et al., 1980), polar lipids (Minnikin et al., 1984) and sugars (Schaal, 1985). The chain lengths of the mycolic acids were determined according to the method described by Linos et al. (1999). The isolate contained the following: meso-diaminopimelic acid, arabinose and galactose (wall chemotype IV sensu Lechevalier & Lechevalier, 1970); N-glycolated muramic acid; a dihydrogenated menaquinone with eight isoprene units [MK-8(H2)] as the predominant menaquinone; mycolic acids within the range C44–C52; and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidyglycerol, phosphatidylinositol and phosphatidylinositol mannosides as major polar lipids (phospholipid type 2; Lechevalier et al., 1977). This chemical profile serves to distinguish the tested strain from members of all of the genera that encompass mycolic-acid-containing organisms (Table 1), apart from representatives of the genus Rhodococcus that have mycolic acids towards the upper carbon range (Goodfellow et al., 1998a).

Strain J81T was also characterized by the presence of large proportions of the fatty acids oleic acid (C18:1; 46.5% of the total), palmitic acid (C16:0; 26.3%) and palmitoleic acid (C16:1; 16.8%) and relatively small proportions of myristic...

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987), based on almost-complete 16S rRNA gene sequences, showing relationships between strains J81T and J82 and representatives of genera classified in the suborder Corynebacterineae. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 75% are shown. Bar, 0.02 substitutions per nucleotide position.
Table 1. Chemical and morphological properties of strain J81T and representatives of genera that contain mycolic-acid-containing organisms

Data for established genera are from Soddell & Seviour (1994), Butler et al. (2005) and Goodfellow & Maldonado (2006). PE indicates the presence (+) or absence (−) of phosphatidylethanolamine. ND, No data.

<table>
<thead>
<tr>
<th>Strain/genus</th>
<th>Major menaquinone</th>
<th>Muramic acid acyl type</th>
<th>Mycolic acids (no. of carbons)</th>
<th>PE</th>
<th>DNA G+C content (mol%)</th>
<th>Cellular micromorphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain J81T</td>
<td>MK-8(H2)</td>
<td>Glycolated</td>
<td>44–52</td>
<td>+</td>
<td>64-7</td>
<td>Characteristic rudimentary right-angled branching</td>
</tr>
<tr>
<td>Dietzia</td>
<td>MK-8(H2)</td>
<td>Acetylated</td>
<td>34–39</td>
<td>+</td>
<td>66–73</td>
<td>Short rods and cocci</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>MK-8(H2)</td>
<td>Acetylated</td>
<td>22–38</td>
<td>−</td>
<td>51–67</td>
<td>Pleomorphic rods</td>
</tr>
<tr>
<td>Gordonia</td>
<td>MK-9(H2)</td>
<td>Glycolated</td>
<td>46–66</td>
<td>+</td>
<td>63–69</td>
<td>Rods and cocci/moderately branched hyphae</td>
</tr>
<tr>
<td>Mycobacterium</td>
<td>MK-9(H2)</td>
<td>Glycolated</td>
<td>60–90</td>
<td>+</td>
<td>62–70</td>
<td>Rods, occasionally branched filaments</td>
</tr>
<tr>
<td>Nocardia</td>
<td>MK-8(H4, co-cycl.)</td>
<td>Glycolated</td>
<td>40–64</td>
<td>+</td>
<td>64–72</td>
<td>Mycelium that fragments into rods and cocci</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>MK-8(H2)</td>
<td>Glycolated</td>
<td>30–54</td>
<td>+</td>
<td>67–73</td>
<td>Rods to extensively branched elements that fragment</td>
</tr>
<tr>
<td>Segnilliparus</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>68–72</td>
<td>Rods</td>
</tr>
<tr>
<td>Skermania</td>
<td>MK-8(H4, co-cycl.)</td>
<td>Glycolated</td>
<td>58–64</td>
<td>+</td>
<td>67–5</td>
<td>Mycelium resembling a pine tree</td>
</tr>
<tr>
<td>Tsukamurella</td>
<td>MK-9</td>
<td>Glycolated</td>
<td>64–78</td>
<td>+</td>
<td>67–74</td>
<td>Rods and coccobacilli</td>
</tr>
<tr>
<td>Williamsia</td>
<td>MK-9(H2)</td>
<td>Glycolated</td>
<td>50–56</td>
<td>+</td>
<td>64–65</td>
<td>Irregular rods or cocci</td>
</tr>
</tbody>
</table>

acid (1·8%), stearic acid (2·3%) and tuberculostearic acid (3·0%). The large proportion of oleic acid and the low proportion of tuberculostearic acid distinguish the organism from representatives of all of the genera composed of mycolic-acid-containing actinomycetes (Kämpfer et al., 1999; Yoon et al., 2000; Gürtler et al., 2001; Kattar et al., 2001; Kim et al., 2002; Linos et al., 2002; Nam et al., 2003; Butler et al., 2005). The highest identification score in the MIDI system (0·041) for the tested strain was with representatives of the genera Dietzia and Rhodococcus, but this score is too low to allow assignment of the strain to either of these genera; consequently, strain J81T cannot be assigned to any of the taxa present in the MIDI database. One-dimensional TLC showed that the organism contained mycolic acids with an Rf value similar to those found for the Gordonia and Rhodococcus marker strains (Rf 0·37–0·38). Strain J81T was also compared with representatives of mycolic-acid-containing genera by using a Curie-point pyrolysis mass spectrometric procedure described by Goodfellow et al. (1998b). It is apparent from Fig. 2 that the organism is well separated from the other strains, as it occupies a distinct area in the resultant taxospace.

The DNA G+C content was determined for strain J81T. Isolation and purification of the DNA was achieved by following the procedure described by Pitcher et al. (1989). The G+C content of the DNA preparation was determined using the reverse-phase HPLC method described by Tamaoka (1994). The molar G+C content, calculated using the method described by Mesbah et al. (1989), was 64·7 mol%.

It is apparent from the excellent congruence found between the present chemotaxonomic and earlier numerical phenetic data (Soddell et al., 1998) that strain J81T should be classified in a novel genus within the suborder Corynebacterineae. This conclusion is strongly supported by the 16S rRNA sequence data, which show that strains J81T and J82 form a distinct
lineage in the Corynebacterineae gene tree, and by the distinctive cellular morphology of these organisms (Soddell & Seviour, 1994). It is proposed that these strains be classified in a novel monospecific genus. The name proposed for this taxon is Millisia brevis gen. nov., sp. nov.

Members of mycolic-acid-containing genera have been assigned to six suprageneric taxa, the families Corynebacteriaceae, Dietziaceae, Gordoniaceae, Mycobacteriaceae, Nocardiaceae and Tsukamurellaceae, mainly on the basis of 16S rRNA signature nucleotides (Stackebrandt et al., 1997; Goodfellow & Maldonado, 2006). The Millisia strains have signature nucleotides that are characteristic of the subclass Actinobacteridae and of the suborder Corynebacterineae, but not of those of their nearest phylogenetic neighbours in the family Gordoniaceae. Further comparative taxonomic studies on additional Millisia strains are needed to determine whether this taxon should be assigned to a novel family. An organism associated with a deep-water marine invertebrate has been found to share an identical partial 16S rRNA gene sequence with M. brevis J81T (Sfanos et al., 2005).

Description of Millisia gen. nov.

Millisia (Mill’i-si.a. N.L. fem. n. Millisia named after Professor Emeritus Nancy F. Millis AC, MBE, a celebrated Australian microbiologist who promoted wastewater microbiology in Australia).

The description is based on data taken from this and previous studies (Soddell & Seviour, 1998; Soddell et al., 1998). Aerobic, Gram-positive to Gram-variable, acid–alcohol-fast, non-motile, catalase-positive actinomycetes that form non-sporo-forming rods showing rudimentary right-angled branching and which contain polyphosphate storage granules. Form salmon-pink, irregular colonies with filamentous margins and sparse, unbranched aerial hyphae on glucose/yeast extract agar. Colonies are matt and dry in appearance, soft in texture and easy to emulsify. Diffusible pigments are not formed. Whole-organism hydrolysates are rich in meso-diaminopimelic acid, arabinose and galactose. Contain N-glycolated muramic acid residues. The predominant menaquinone has eight isoprene units, one of which is hydrogenated. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylglycositol mannosides are the major polar lipids. The mycolic acids present have 44–52 carbons (principal components C_{40}, C_{50} and C_{52}). Oleic, palmitic and palmitoleic acids are the predominant fatty acids, and there are relatively small proportions of myristic, stearic and tuberculostearic acids. The G+C content of the genomic DNA of the type strain of the type species is 64·7 mol%. The genus forms a distinct lineage in the Corynebacterineae 16S rRNA gene tree. The type species is Millisia brevis.

Description of Millisia brevis sp. nov.

Millisia brevis (bre’vis. L. fem. adj, brevis short, denoting the formation of short, branched rods).

The description is based upon data taken from Soddell et al. (1998). In addition to having the properties given in the genus description, the organism degrades Tweens 20, 40 and 60, but not adenine, casein, elastin, hypoxanthine, tyrosine or xanthine. Hydrolyses allantoin (weakly) and urea (strongly) but does not reduce nitrate or produce m- or p-nitrophenol oxides. Grows between 15 and 35 °C, but not at 10 or 37 °C, and at pH 5·5–9·5. Cellobiose, ethanol, fructose, glucose, glycerol, myo-inositol, mannitol, mannos, sucrose and trehalose are used as sole sources of carbon for energy and growth, but arabinose, galactose, inulin, lactose, maltose, melezitose, raffinose, rhamnose, salicin, sorbitol and xylose (all at 0·1%, w/v) are not. Similarly, m-hydroxybenzoic acid, sebacic acid, sodium butyrate, sodium gluconate, sodium lactate, sodium octanoate and sodium pyruvate are used as sole carbon sources, but acetamide, benzamide, p-hydroxybenzoic acid, pimelic acid, sodium benzoate, sodium succinate, sodium tartrate, testosterone and tyrosine (0·01%, w/v) are not; variable results are obtained with p-cresol, sodium acetate, sodium adipate, sodium citrate, sodium fumarate, sodium malate and sodium propionate (all at 0·01%, w/v). Coconut oil, glycerol trioleate, kerosene, hexadexane, olive oil, paraffin oil, safflower oil and xylene (weakly) are used as sole carbon sources in shake-flask liquid culture (all at 1%, v/v).

Variable results are obtained with acetamide and serine as sole carbon and nitrogen sources, but trimethylamine does not support growth. Grows in the presence of crystal violet (0·0001%, w/v), phenol (0·01%, w/v) and phenyl ethanol (0·02 and 0·03%, v/v), but is sensitive to crystal violet (0·001%, w/v), phenol (0·1%, w/v), sodium azide (0·01 and 0·02%, w/v), sodium chloride (5 and 7%, w/v), and penicillin G (10 IU). Produces acid phosphatase, alkaline phosphatase (weakly), esterase (C_{4}), esterase lipase (C_{6}), a-glucosidase, b-glucosidase, leucine arylamidase, a-mannosidase (weakly), naphthol-AS-BI-phosphohydrolase and valine arylamidase, but not chymotrypsin, lipase (C_{14}), a-fucosidase, a-galactosidase, b-galactosidases, b-glucuronidase, N-acetyl-b-glucosaminidase or trypsin; variable results are obtained for cystine arylamidase.

The type strain, strain J81T (=DSM 44463T = NRRL B-24424T), was isolated from activated sludge foam from Tamworth Sewage Treatment Plant, New South Wales, Australia.

References


