Amycolicicoccus subflavus gen. nov., sp. nov., an actinomycete isolated from a saline soil contaminated by crude oil

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Two novel actinomycetes, designated DQS3-9A1T and DQS3-9A2, were isolated from a saline soil contaminated with crude oil in the Shengli Oilfield in China. On the basis of 16S rRNA gene sequence analysis, the two strains were most closely related to Mycobacterium species (92.7–94.9 % similarities), and formed a distinct lineage in the suborder Corynebacterineae. In addition, the major sugars in the cell wall, arabinose and galactose, supported the affiliation of strain DQS3-9A1T with members of the family Mycobacteriaceae. However, strain DQS3-9A1T did not contain mycolic acids and MK-8 (85.5 %) was the major menaquinone for both isolates. The major cellular fatty acids for strain DQS3-9A1T were C16 : 0 (20.5 %), 10-methyl C17 : 0 (19.3 %), 10-methyl C18 : 0 (16.1 %), summed feature 3 (11.4 %), C15 : 0 (11.3 %), C17 : 0 (5.0 %) and C17 : 1ω8c (5.0 %). The polar lipids of strain DQS3-9A1T consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol and an unknown glucosamine-containing phospholipid. These chemotaxonomic data indicated that strain DQS3-9A1T differs from the present members of the suborder Corynebacterineae. Therefore, the creation of Amycolicicoccus subflavus gen. nov., sp. nov. is proposed, with DQS3-9A1T (=DSM 45089T=CGMCC 4.3532T) as the type strain.

The suborder Corynebacterineae was first described by Stackebrandt et al. (1997) and comprises seven families, Nocardiaceae, Gordoniaceae, Tsukamurellaceae, Dietziaceae, Corynebacteriaceae, Mycobacteriaceae (Stackebrandt et al., 1997) and Segniliparaceae (Butler et al., 2005). Each family encompasses one genus, except Nocardiaceae, which contains the genera Nocardia and Rhodococcus, and Gordoniaceae, which contains the genera Gordonia, Millisia (Soddell et al., 2006) and Skermania. Currently, the suborder Corynebacterineae comprises these 10 genera and another genus, Williamsia (Kämpfer et al., 1999). Members of these genera form distinct lineages in the phylogenetic tree based on 16S rRNA gene sequences. Almost all members of the suborder Corynebacterineae contain mycolic acids, except for Corynebacterium amycolatum (Collins et al., 1988), Corynebacterium atypicum (Hall et al., 2003), Corynebacterium caspium (Collins et al., 2004), Corynebacterium iconiae (Fernández-Garayzábal et al., 2004), Corynebacterium kroppenstedtii (Collins et al., 1998), Corynebacterium minutissimum, Corynebacterium striatum and Corynebacterium xerosis (Wauters et al., 1996).

Strains DQS3-9A1T and DQS3-9A2 were isolated from a saline soil contaminated with crude oil in the Shengli Oilfield of eastern China by 10-fold dilution plating on agar plates containing only oil-produced water at 30 °C for 7 days. The two isolates were purified and incubated on plates of artificial seawater (ASW) agar (1%: peptone, 5 g; yeast extract, 1 g; Na2SO4, 4 g; KCl, 0.68 g; KBr, 0.1 g; H3BO3, 0.025 g; MgCl2.6H2O, 5.4 g; CaCl2.2H2O, 1.5 g; SrCl2.6H2O, 0.024 g; NaHCO3, 0.2 g; Na2HPO4, 0.04 g; NH4Cl, 0.5 g; NaF, 0.002 g; pH 8.0) with 2.4 % NaCl (Eguchi et al., 1996) for 2 days at 30 °C. Growth was assessed at different temperatures (5–50 °C), pH values (2.0–12.0) and salinity (0–20 %, w/v, NaCl). The pH tolerance was examined in ASW medium containing 5 %
(w/v) NaCl at 30 °C. The NaCl concentration required for growth was investigated between 0 and 20 % (w/v) in ASW medium at pH 7.0 and 30 °C.

Morphological characteristics of strain DQS3-9A1T were examined using scanning electron microscopy. Denitrification was assessed using a previously described method (Zumft, 1991). Hydrolysis of starch, gelatin and Tween 80, along with urease activity and carbon source utilization, were examined according to Williams et al. (1983) on ASW medium after 5–7 days incubation at 30 °C. Cellular fatty acid methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). Fatty acid analysis was performed using the Sherlock system (Microbial ID). Isoprenoid quinones were analysed using HPLC with a reversed-phase column (Shim-pack, VPODS; Shimazu) as described by Komagata & Suzuki (1987). Polar lipids were extracted and examined by one- and two-dimensional TLC on Merck silica gel 60 F254 aluminium-backed thin-layer plates according to the procedures described by Kates (1986) and Collins et al. (1980).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Wang et al. (2007). The G+C content of genomic DNA was determined by thermal denaturation (Marmur & Doty, 1962) with DNA from Escherichia coli K-12 as a control. DNA–DNA hybridization was carried out in triplicate following the methods of De Ley et al. (1970) and Huß et al. (1983). The 16S rRNA gene sequence was compared to sequences obtained from public databases (GenBank/EMBL/DDBJ) to find the most closely related species. Phylogenetic analysis was performed using the software package MEGA version 3.1 (Kumar et al., 2004) after multiple sequence alignment with CLUSTAL_X (Thompson et al., 1997). A phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) based on a distance matrix that was corrected by Kimura’s two-parameter model (Kimura, 1980). Bootstrap analysis with 1000 resamplings was used to evaluate the robustness of the tree’s topology (Felsenstein, 1985).

Both strain DQS3-9A1T and strain DQS3-9A2 were Gram-positive cocci. The cells of strain DQS3-9A1T were 0.7–0.9×0.9–1.05 μm without a flagellum (Fig. 1). Neither strain formed spores. The colonies of the two strains were dry, opaque and light-yellowish white on ASW agar. Their growth occurred at 15–42 °C, pH 6.0–11.0 and in 1–12 % (w/v) NaCl. Tests of strains DQS3-9A1T and DQS3-9A2 for hydrolysis of starch, gelatin, Tween 80 and casein, production of indole and DNase, aerobic nitrite reduction and anaerobic nitrate reduction were negative, whereas tests for hydrogen sulfide production and aerobic nitrate reduction were positive. The utilization pattern of sole carbon and nitrogen sources of strains DQS3-9A1T and DQS3-9A2 was identical. Detailed physiological characteristics of isolate DQS3-9A1T are given in the species description.

The almost-complete 16S rRNA gene sequences of strains DQS3-9A1T and DQS3-9A2 were obtained. In the phylogenetic tree (Fig. 2), the two strains clustered in a distinct clade that was strongly supported by the high bootstrap value (100 %), and the clade could be equated with branches corresponding to recognized genera. The strains tested were most closely related to recognized Mycobacterium species (92.7–94.9 % similarities). Additionally, the major sugars in the cell wall of the two strains were arabinose and galactose, also supporting the affiliation of the isolates to the family Mycobacteriaceae. The predominant menaquinones of strain DQS3-9A1T were MK-8 (85.5 %) and MK-7 (8.0 %), and the major cellular fatty acids were C₁₆:0 (20.5 %), 10-methyl C₁₇:0 (19.3 %), 10-methyl C₁₈:0 (16.1 %), summed feature 3 (11.4 %), C₁₅:0 (11.3 %), C₁₇:0 (5.0 %) and C₁₇:1ω8c (5.0 %). The patterns of the predominant menaquinones and major cellular fatty acid composition of strain DQS3-9A2 were identical to those of strain DQS3-9A1T. The polar lipids of strain DQS3-9A1T consisted of diphosphatidyglycerol, phosphatidylethanolamine, phosphatidyglycerol, phosphatidylcholine, phosphatidylinositol and an unknown glucosamine-containing phospholipid. Mycolic acid, which is the most important character shared by genera in the Corynebacterineae, could not be detected in strain DQS3-9A1T. The above chemical profiles therefore served to distinguish the tested strains from members of all genera in the Corynebacterineae (Table 1). Furthermore, distinctions could be made between strains DQS3-9A1T and DQS3-9A2 and other members of the family Mycobacteriaceae in some signature nucleotide sites (Table 2). The DNA G+C content of strain DQS3-9A1T was 60.04 mol%. Other biochemical characteristics of isolate DQS3-9A1T are given in the genus description.

As a result, based on 16S rRNA gene sequence analysis, phenotypic characteristics and chemotaxonomic prop-
It is clear that strains DQS3-9A1<sup>T</sup> and DQS3-9A2 represent a novel genus within the family Mycobacteriaceae of the suborder Corynebacterineae, for which the name Amycolicicoccus subflavus gen. nov., sp. nov. is proposed.

**Description of Amycolicicoccus gen. nov.**

*Amycolicicoccus* [A.my.co.li.ci.coc’cus. Gr. pref. a- not; N.L. n. acidum mycolicum mycolic acid; N.L. masc. n. coccus (from Gr. n. kokkos) a grain, berry; N.L. masc. n. *Amycolicicoccus* a coccus without mycolic acids].

Gram-positive, aerobic cocci without a flagellum. Spores are not present. Cell wall contains arabinose, galactose, glucose and xylose as major sugars, and alanine, glutamic acid, methionine and histidine as major amino acids. The polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol and an unknown glucosamine-containing phospholipid. The predominant menaquinones are MK-8 and MK-7, and the major cellular fatty acids are C<sub>16:0</sub>, 10-methyl C<sub>17:0</sub>, 10-methyl C<sub>18:0</sub> summed feature 3, C<sub>15:0</sub>, C<sub>17:0</sub> and C<sub>17:1ω6c</sub>. Mycolic acid is absent. The type species is *Amycolicicoccus subflavus*.

**Description of Amycolicicoccus subflavus**

*Amycolicicoccus subflavus* (sub.flav.’us. L. masc. adj. sub-flavus yellowish).

Displays the following properties in addition to those described for the genus. Cells are 0.7–0.9 × 0.9–1.05 μm without a flagellum and colonies are dry, opaque and light-yellowish white on ASW agar. Growth occurs at 15–42 °C (optimum, 37 °C), pH 6.0–11.0 (optimum, pH 8.0) and in 1–12 % (w/v) NaCl [optimum, 8 % (w/v) NaCl]. Negative for hydrolysis of starch, gelatin, Tween 80 and casein, production of indole and DNase, aerobic nitrite reduction and anaerobic nitrate reduction. Positive for production of hydrogen sulfide and aerobic nitrate reduction. Cellobiose, dextrin, fucose, glucose, inositol, malic acid, maltose, mannose, mannitol, proline, sodium gluconate, sorbitol, sucrose and succinic acid are utilized as sole carbon and nitrogen sources, but alanine, arabinose, arginine, fructose, galactose, lactose, malonic acid, ribose, sorbose and xylose are not. Resistant to gentamicin (10 μg), clindamycin (2 μg), sulfamethoxazole (300 μg), streptomycin (300 μg) and amikacin (30 μg). Sensitive to cephalothin (30 μg), cefotaxime (30 μg) and penicillin G (30 μg).
Table 1. Chemical and morphological properties of strain DQS3-9A1<sup>T</sup> and other genera of the suborder Corynebacterineae

1, DQS3-9A1<sup>T</sup>; 2, Mycobacterium; 3, Corynebacterium; 4, Segniliparus; 5, Dietzia; 6, Tsukamurella; 7, Skermania; 8, Millisia; 9, Gordonia; 10, Rhodococcus; 11, Nocardia; 12, Williamsia. Data from this study and Butler et al. (2005), Chun et al. (1997), Kämpfer et al. (1999), Li (2007), Rainey et al. (1995) and Soddell et al. (2006).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Cocci</td>
<td>Rods, occasionally branched filaments</td>
<td>Pleomorphic rods</td>
<td>Rods</td>
<td>Short rods and cocci</td>
<td>Rods and coccobacilli</td>
<td>Mycelium resembling a pine tree</td>
<td>Characteristic rudimentary right-angled branching</td>
<td>Rods and cocci/moderately branched hyphae</td>
<td>Rods to extensively branched elements that fragment</td>
<td>Mycelium that fragments into rods and cocci</td>
<td>Irregular rods or cocci</td>
</tr>
<tr>
<td>Aerial hyphae</td>
<td>Absent</td>
<td>Usually absent</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Mycolic acid</td>
<td>Absent</td>
<td>Present</td>
<td>MK-9(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>ND</td>
<td>MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>MK-9</td>
<td>Present</td>
<td>MK-8(H&lt;sub&gt;4&lt;/sub&gt;, co-cycl.)</td>
<td>Absent</td>
<td>Present</td>
<td>MK-8(H&lt;sub&gt;4&lt;/sub&gt;, co-cycl.)</td>
</tr>
<tr>
<td>Predominant menaquinone</td>
<td>MK-8</td>
<td>MK-8(H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>ND</td>
<td>DPG, PE, PG, PI, PIM</td>
<td>DPG, PG</td>
<td>DPG, PE, PG</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI, PIM</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>60.04</td>
<td>62–70</td>
<td>51–67</td>
<td>68–72</td>
<td>66–73</td>
<td>67–74</td>
<td>67.5</td>
<td>64.7</td>
<td>63–69</td>
<td>67–73</td>
<td>64–72</td>
<td>64–65</td>
</tr>
</tbody>
</table>

ND, No data.

*DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidyglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; GluNU, unknown glucosamine-containing phospholipid; PIM, phosphatidylinositol mannosides.
†S, Saturated fatty acids; U, unsaturated fatty acids; T, tuberculostearic acid; SF3, summed feature 3 (16:1<sup>07c/15</sup> iso 2OH).
‡Tuberculostearic acid present in Corynebacterium ammoniagenes, Corynebacterium bovis, Corynebacterium minutissimum, Corynebacterium urealyticum and Corynebacterium variabilis.
Table 2. Selected 16S rRNA gene signature nucleotides detected for the family Mycobacteriaceae, strain DQS3-9A1T and strain DQS3-9A2

The 16S rRNA gene sequences of all type strains belonging to the family Mycobacteriaceae were included in this analysis. The signatures given below for each group were chosen for their presence in more than 95% of the members of that group.

<table>
<thead>
<tr>
<th>Position(s)</th>
<th>Mycobacteriaceae</th>
<th>DQS3-9A1T</th>
<th>DQS3-9A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>293–304</td>
<td>G–U</td>
<td>G–C</td>
<td>G–C</td>
</tr>
<tr>
<td>307</td>
<td>A</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>316–337</td>
<td>U–G</td>
<td>C–G</td>
<td>G–C</td>
</tr>
<tr>
<td>468</td>
<td>U</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>508</td>
<td>U</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>631</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>661–744</td>
<td>G–C</td>
<td>G–C</td>
<td>G–C</td>
</tr>
<tr>
<td>662–743</td>
<td>U–G</td>
<td>C–G</td>
<td>C–G</td>
</tr>
<tr>
<td>771–808</td>
<td>A–U</td>
<td>G–C</td>
<td>G–C</td>
</tr>
<tr>
<td>824–876</td>
<td>C–G</td>
<td>U–A</td>
<td>U–A</td>
</tr>
<tr>
<td>825–875</td>
<td>G–C</td>
<td>A–U</td>
<td>A–U</td>
</tr>
<tr>
<td>837–849</td>
<td>G–U</td>
<td>U–A</td>
<td>U–A</td>
</tr>
<tr>
<td>843</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>1059–1198</td>
<td>U–A</td>
<td>C–G</td>
<td>C–G</td>
</tr>
</tbody>
</table>

ciprofloxacin (5 μg), ceftriazone (30 μg), penicillin (10 IU), clarithromycin (15 μg), chloramphenicol (30 μg), erythromycin (15 μg), cefotaxime (30 μg), vancomycin (30 μg) and ampicillin (10 μg). The DNA G+C content is 60.04 mol%.

The type strain, DQS3-9A1T (=DSM 45089T=CGMCC 4.3532T), was isolated from an oil-polluted saline soil in Shengli Oilfield, eastern China. DQS3-9A2, isolated from the same site, is a reference strain.

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