Demequina aestuarii gen. nov., sp. nov., a novel actinomycete of the suborder Micrococcciniae, and reclassification of Cellulomonas fermentans Bagnara et al. 1985 as Actinotalea fermentans gen. nov., comb. nov.

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An actinobacterial strain containing demethylmenaquinone DMK-9(H4) as the diagnostic isoprenoid quinone was isolated from a tidal flat sediment sample, from South Korea. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain JC2054T represents a distinct phyletic line within the suborder Micrococcciniae of the order Actinomycetales. The closest phylogenetic neighbour was Cellulomonas fermentans, with 94.7 % 16S rRNA gene sequence similarity. The novel isolate was strictly aerobic and slightly halophilic, with optimum growth occurring in 2–4 % (w/v) NaCl. Cells were non-motile, non-sporulating and rod-shaped. The peptidoglycan type was of the A-type of cross-linkage. L-Omehine was the diamino acid and D-glutamate represented the N-terminus of the interpeptide bridge. The predominant fatty acids were anteiso-branched and straight-chain fatty acids. The major polar lipids were phosphatidylinositol, diphosphatidylglycerol and an unknown phospholipid. The menaquinone composition of C. fermentans was determined to be MK-10(H4), MK-9(H4) and MK-8(H4) in the ratio 56 : 2 : 1. On the basis of the polyphasic evidence presented in this study, it is proposed that strain JC2054T should be classified as representing a novel genus and species of the suborder Micrococcciniae, with the name Demequina aestuarii gen. nov., sp. nov. The type strain is JC2054T (=IMSNU 14027T = KCTC 9919T = JCM 12123T). In addition, it was clear from the phylogenetic analysis and chemotaxonomic data that C. fermentans does not belong to the genus Cellulomonas or any other recognized genera. Therefore, C. fermentans should be reclassified as representing a novel genus, for which the name Actinotalea fermentans gen. nov., comb. nov. is proposed, with strain DSM 3133T (=ATCC 43279T = CFBP 4259T = CIP 103003T = NBRC 15517T = JCM 9966T = LMG 16154T) as the type strain.

The west and south-west coasts of the Korean peninsula consist mainly of tidal flats that are unique among other marine sediments. The high microbial diversity of Korean tidal flat sediments has been reported in culture-independent (Kim et al., 2004, 2005) and culture-dependent (Park et al., 2005; Yi & Chun, 2006) studies. A novel actinomycete was isolated from Korean tidal flat sediment and subjected to a comprehensive taxonomic study. Here, we report the taxonomic description of this strain.

An actinomycete strain, designated JC2054T, was isolated from a tidal flat sediment sample from Ganghwa Island, South Korea (37° 36′ 22.3″ N 126° 22′ 59.4″ E), using the standard dilution plating method based on marine agar 2216 (MA; Difco). The isolate was routinely cultured on MA and maintained as a glycerol suspension (20 %, w/v) at −80 °C.

The 16S rRNA gene was amplified enzymically from a single colony. The primers, PCR conditions and sequencing methods used have been described elsewhere (Chun & Goodfellow, 1995). Preliminary sequence comparison against 16S rRNA gene sequences held in the GenBank database indicated that our isolate belonged to the suborder Micrococcciniae in the order Actinomycetales. The nearly complete 16S rRNA gene sequence of strain JC2054T (1423 bp) was aligned manually against those of...
representatives of the suborder *Micrococcineae* using the bacterial 16S rRNA secondary structure model. The regions available for all sequences (positions 45–71 and 98–1410; *Escherichia coli* numbering system) showed unambiguous alignment and were used to construct phylogenetic trees. The phylogenetic trees were inferred by using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (Jeon *et al.*, 2005; available at http://chunlab.snu.ac.kr/jphydit) and PAUP 4.0 (Swofford, 1998), as described previously (Yi & Chun, 2006).

On the basis of 16S rRNA gene sequence analyses, strain JC2054\textsuperscript{T} showed the highest sequence similarity to *Cellulomonas fermentans* DSM 3133\textsuperscript{T} (94.7 %) and *Sanguibacter* species (94.1–94.7 %). No other taxa showed more than 94 % sequence similarity to our isolate. The close relationship between strain JC2054\textsuperscript{T} and other members of the suborder *Micrococcineae* was also evident from the phylogenetic tree (Fig. 1). Strain JC2054\textsuperscript{T} formed a clade with *C. fermentans* in the neighbour-joining (with 64 % bootstrap support), maximum-likelihood and maximum-parsimony trees, and was recovered as a sister group of the genus *Sanguibacter* in the Fitch–Margoliash tree. Apart from the close relationship with strain JC2054\textsuperscript{T}, *C. fermentans* showed the highest sequence similarities to the genera *Cellulomonas* (94.4–95.8 %), *Oerskovia* (94.2–94.7 %), *Tetrasphaera* (94.6 %) and *Sanguibacter* (94.1–94.4 %). It is evident from the phylogenetic tree (Fig. 1) that our isolate together with *C. fermentans* are not members of the genus *Cellulomonas*.

Cellular morphology and motility were examined by scanning-electron and phase-contrast microscopy, respectively, using cells that had been grown on MA at 30 °C for 3 days. The presence of mycelium and spore formation were examined following growth at 30 °C for 3 weeks on several culture media: ISP medium No. 3 (Difco) supplemented with 4 % (w/v) sea salts (Sigma), ISP medium No. 4 (Difco) supplemented with 4 % (w/v) sea salts (Sigma), ISP medium No. 4 (Difco).
supplemented with sea salts, PYGV (Staley, 1968) supplemented with sea salts, basal medium (BM; Baumann et al., 1972) and MA.

Growth under anaerobic conditions was checked using MA, in an anaerobic chamber [10 % CO₂, 10 % H₂, 80 % N₂ (v/v); Sheldon Manufacturing]. The temperature range for growth (5–50 °C), the pH range (pH 4–12, adjusted with 10 M KOH and HCl) and tolerance of NaCl (0–15 %, w/v) were determined using synthetic Zobell medium [Zobell, 1941; 5 g Bacto peptone (Difco), 1 g yeast extract (Difco), 0.1 g ferric citrate, per litre seawater] for up to 3 weeks. The optimal temperature for growth was also determined by using a temperature-gradient incubator (TVS 126 MA; Advantec).

Standard physiological and biochemical tests were performed as described by Smibert & Krieg (1994). The acid-fast test was performed using Ziehl-Neelsen staining. Hydrolysis of alginic acid, casein, carboxymethylcellulose, chitin, starch and Tween 80 was tested using MA as the basal medium. DNase test agar (Difco) supplemented with 2.5 % (w/v) NaCl was used for the DNase assay. Decomposition of adenine, hypoxanthine, L-tyrosine and xanthine was tested using MA, according to Gordon et al. (1974). Production of H₂S was detected using triple sugar iron agar supplemented with 2.5 % (w/v) NaCl. Tests for arginine dihydrolase and β-galactosidase activities, nitrate reduction, urease activity, acid production from glucose and indole production were performed using an API 20NE kit (bioMérieux). Other enzymic activities were determined using an API ZYM kit (bioMérieux). Strips for API kits were inoculated with a heavy bacterial suspension in half-strength artificial seawater or AUX medium (bioMérieux), supplemented with 2.5 % (w/v) NaCl. Carbon source utilization was tested in 96-well tissue culture microplates (Falcon) for up to 7 days using BM supplemented with 1 % (v/v) vitamin solution (Staley, 1968) as basal medium.

The results of the morphological, cultural, biochemical and physiological tests are given in the genus and species description.

Biomass for chemotaxonomy was scraped from MA plates. Extraction and gas chromatographic separation of fatty acid methyl esters were performed using the Microbial Identification System (MIDI), according to the instructions of the manufacturer. Peptidoglycan was purified and analysed according to Schleifer & Seidl (1985). Amino acids and peptides of peptidoglycan hydrolysates were separated by two-dimensional ascending TLC on cellulose plates, using the solvent systems of Schleifer & Kandler (1972). The molar ratios of amino acids were determined by GC and GC/MS of N-heptafluorobutyryl amino acid isobutyl esters (Groth et al., 1996; MacKenzie, 1987). The amino terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). The glycolate content of bacterial cell walls was determined by using the colorimetric method of Uchida & Aida (1984). Menaquinones were extracted and purified according to Collins et al. (1977) and analysed by using a modification of the HPLC method described by Groth et al. (1996) [isocratic elution from an RP 18 column (no. 201HS5415; Vydac) using acetonitrile/2-propanol (65 : 35, v/v) at 35 °C] and electron-impact mass spectrometry as described by Pukall et al. (2006). The DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah et al. (1989). Phospholipids were extracted, purified and identified as described by Yassin et al. (1993).

The peptidoglycan of strain JC2054ᵀ was composed of L-ornithine, alanine, glycine, serine, D-glutamate and aspartate in an approximate molar ratio of 1.0 : 1.2 : 1.4 : 1.7 : 5.5 : 0.4. Glutamic acid represented the N-terminus of the interpeptide bridge. The cross-linkage was of the A-type, as the characteristic peptide was L-Ala→D-Glu (Schleifer & Kandler, 1972). L-Ornithine was the diamino acid. The major polar lipids were phosphatidilinositol, diphasphatidylglycerol and an unknown phospholipid. The fatty acid composition of strain JC2054ᵀ was as follows: C₁₄ : 0 (1.59 %), C₁₅ : 0 (4.16 %), C₁₆ : 0 (10.42 %), C₁₇ : 0 (0.70 %), i-C₁₄ : 0 (1.08 %), i-C₁₅ : 0 (3.31 %), i-C₁₆ : 0 (6.96 %), i-C₁₇ : 0 (0.91 %), ai-C₁₅ : 0 (46.83 %), ai-C₁₇ : 0 (13.14 %), i-C₁₅ : 1 (0.68 %) and ai-C₁₅ : 1 (10.21 %). The DNA G+C content was 67 mol%. HPLC analysis of the menaquinone composition of strain JC2054ᵀ revealed a single component that eluted at a retention time that was earlier by 10 % than that of MK-9(H₄) extracted from Oerskovia turbata DSM 20577ᵀ. The electron-impact mass spectrum of the isoprenoid quinone of strain JC2054ᵀ clearly showed a base peak at m/z 211, and the peak of the molecular ion at m/z 775. The corresponding values for MK-9(H₄) are m/z 225 and 788, respectively. Taking an instrumental error of approximately 1 mass unit into account, this result suggests the presence of demethylmenaquinone DMK-9(H₄) in strain JC2054ᵀ (Collins, 1994). In contrast, the isoprenoid quinone composition of C. fermentans DSM 3133ᵀ was MK-10(H₄), MK-9(H₄) and MK-8(H₄) in the ratio 56 : 2 : 1, as revealed in this study. These results differentiate C. fermentans from strain JC2054ᵀ, as well as from bona fide members of the genus Cellulomonas that contain MK-9(H₄) as the major isoprenoid quinone (Stackebrandt et al., 2002).

**Taxonomic conclusions**

Phylogenetic analyses based on 16S rRNA gene sequences showed that strain JC2054ᵀ represents a distinct phyletic line within the suborder Micrococccinae of the order Actinomycetales. The highest sequence similarity values to our isolate were observed for C. fermentans DSM 3133ᵀ (94.7 %) and members of the genus Sanguibacter (94.1–94.7 %). However, strain JC2054ᵀ could be clearly differentiated from all the phylogenetically related genera by the presence of DMK-9(H₄), which hitherto has not been detected as a major menaquinone of members of the suborder Micrococccinae, and by several other chemotaxonomic characteristics (Table 1). It is therefore proposed
Table 1. Major chemotaxonomic characteristics that differentiate strain JC2054T from phylogenetically related bacteria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain JC2054T</th>
<th>Cellulomonas fermentans</th>
<th>Cellulomonas</th>
<th>Cellulosimicrobium</th>
<th>Intrasporangium Isoptericola</th>
<th>Terrabacter Terracoccus</th>
<th>Tetrasphaera</th>
<th>Ornithinicoccus</th>
<th>Terrabacter</th>
<th>H. Yi, P. Schumann and J. Chun</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major fatty acids</td>
<td>D-Asp or D-Glu</td>
<td>D-Asp or D-Glu</td>
<td>D-Asp</td>
<td>D-Asp or D-Glu</td>
<td>D-Asp or D-Glu</td>
<td>D-Asp or D-Glu</td>
<td>D-Asp</td>
<td>D-Asp</td>
<td>D-Asp</td>
<td>D-Asp</td>
</tr>
<tr>
<td>Interpeptide bridge</td>
<td>L-Thr or L-Thr</td>
<td>L-Thr or L-Thr</td>
<td>L-Thr</td>
<td>L-Thr or L-Thr</td>
<td>L-Thr or L-Thr</td>
<td>L-Thr or L-Thr</td>
<td>L-Thr</td>
<td>L-Thr</td>
<td>L-Thr</td>
<td>L-Thr</td>
</tr>
<tr>
<td>DNA G+C (mol%)</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
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<tr>
<td>Major menaquinone</td>
<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
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<td>MK-9(H4) meso-A2pm L-Orn LL-A2pm</td>
</tr>
</tbody>
</table>

* A, aerobic; F, facultatively aerobic; S, strictly aerobic.

that the novel isolate should be assigned as representing a novel genus and species in the suborder Micrococccineae, with the name Demequina aestuarii gen. nov., sp. nov. In addition, on the basis of 16S rRNA gene phylogeny (Fig. 1) and chemotaxonomy (Table 1), it is evident that C. fermentans is not a member of the genus Cellulomomas or the newly proposed genus Demequina. Therefore it is proposed that Cellulomonas fermentans should be reclassified as representing a novel genus, with the name Actinotalea fermentans gen. nov., comb. nov.

**Description of Demequina gen. nov.**

Demequina (De.me.qui.na. N.L. fem. n. Demequina arbitrary name derived from demethylmenaquinone, an unusual quinone found in this organism).

Gram-positive, strictly aerobic and slightly halophilic. Oxidase-negative, catalase-positive and acid-fast-negative. Do not form spores. Cells are non-motile rods with round ends. The only isoprenoid quinone is demethylmenaquinone DMK-9(H4). The peptidoglycan type is of the A-type of cross-linkage and contains L-ornithine, alanine, glycine, serine, D-glutamate and aspartate. L-Ornithine is the diamino acid and D-glutamate represents the N-terminus of the interpeptide bridge. Predominant cellular fatty acids are of the anteiso-branched and straight-chain types. Major polar lipids are phosphatidylinositol, diphasphatidylglycerol and an unknown phospholipid. Phylogenetically, the genus is affiliated to the suborder Micrococccineae. The type species is Demequina aestuarii.

**Description of Demequina aestuarii sp. nov.**

Demequina aestuarii (ae.stu.a’ri.i. L. gen. n. aestuarii of a tidal flat, isolated from tidal flat sediment).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.25–0.35 × 0.60–1.20 μm. Growth occurs at pH 6–11 (optimum, pH 7) and in 0–12 % (w/v) NaCl (optimum, 2–4 %). Growth occurs at 5–35 °C, with optimum growth at 33.5 °C. Minimum doubling time is 6.4 h. Extended incubation (up to 15 days) is required at 5 °C. Growth occurs on MA, ISP medium No. 3 supplemented with sea salts, ISP medium No. 4 supplemented with sea salts and PYGV supplemented with sea salts, but not on Oatmeal agar supplemented with sea salts. Colonies on MA at 30 °C are yellow, translucent and circular, with entire margins, approximately 0.2 mm in diameter after 2 days, with a maximum diameter of 3–4 mm after 7 days. Negative for arginine dihydrolase and urease activities. Positive for β-galactosidase activity. Nitrate is not reduced to nitrite. Does not produce H2S. Acid is not produced from glucose nor indole from tryptophan. Decomposes carboxymethylcellulose, DNA and aesculin, but not adenine, alginate, casein, chitin, gelatin, hypoxanthine, starch, Tween 80, tyrosine or xanthine. On the basis of the API ZYM system, the organism is positive for leucine arylamidase, β-galactosidase, α-glucosidase and...
β-glucosidase; weakly positive for naphthol-AS-BI-phosphohydrolase; negative for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannnosidase and α-fucosidase. Utilizes D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, D-salicyl, D-trehalose, D-xylene, glycerol, L-arabinose and sucrose as a sole carbon source. Utilizes lactose weakly. Does not utilize acetamide, acetate, benzoate, citrate, D-ribofus, D-ribose, D-sorbos, ethanol, glycine, inositol, inulin, 2-propanol, L-arginine, L-asparbate, L-asparagine, L-lysine, L-ornithine, L-rafoside, N-acetylglucosamine, polyethylene glycol, salicylate, succinate, tartrate or thiamine. Major fatty acids are ai-C15:0, ai-C17:0, C16:0 and ai-C15:1.

The DNA G+C content of the type strain is 67 mol%. The type strain is JC2054 T (=IMNSU 14027 T =KCTC 9919 T =JCM 12123 T), isolated from a tidal flat sediment sample from Ganghwa Island, South Korea.

**Description of Actinotalea gen. nov.**

*Actinotalea* (Ac.ti.no.tal’e.a. Gr. n. actis, actinis ray; L. fem. n. talea a slender staff, rod, stick; N.L. fem. n. Actinotalea ray stick).

The description is based on that given for *Cellulomonas fermentans* by Bagnara *et al.* (1985). Gram-positive. Coryneform rods exhibiting polymorphism. Non-motile. Facultatively anaerobic. Diamino acid of the peptidoglycan is L-ornithine and interpeptide bridge is D-aspartate. Major fatty acids are C14:0 ai-C15:0 and C16:0. The major isoprenoid quinone is MK-10(H4). The type species is *Actinotalea fermentans*.

**Description of Actinotalea fermentans sp. nov., comb. nov.**


The description is identical to that given for *Cellulomonas fermentans* by Bagnara *et al.* (1985). Cell wall sugars are glucose, rhamnose and ribose. The DNA G+C content of the type strain is 76 mol%. The type strain is DSM 3133 T (=ATCC 43279 T =CFBP 4259 T =CIP 103003 T =NBRC 15517 T =JCM 9966 T =LMG 16154 T).

**References**


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