Three isolates of novel polyphosphate-accumulating Gram-positive cocci, obtained from activated sludge, belong to a new genus, *Tetrasphaera* gen. nov., and description of two new species, *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov.

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Two isolates of Gram-positive cocci (Ben 109T and Ben 110) which could accumulate polyphosphate and were microscopically similar in appearance to so-called ‘G-bacteria’, appearing as tetrads, were isolated from samples of activated sludge biomass by micromanipulation and grown in axenic culture. On the basis of their phenotypic and chemotaxonomic characters and 16S rDNA sequences, these isolates, together with strain T1-X7T isolated and described previously in Japan, belong to a new genus. These isolates are phylogenetically different from *Tessaracoccus bendigoensis*, *Friedmanniella spumicola* and *Friedmanniella capsulata*, Gram-positive cocci isolated previously in this laboratory. They are characterized by type A1γ peptidoglycan, with meso-diaminopimelic acid as the diagnostic diamino acid. The main cellular fatty acid of Ben 109T, Ben 110 and T1-X7T is 14-methylpentadecanoic acid (i-C16:0). The major menaquinones of Ben 109T are MK-8(H4), with MK-8(H2) and MK-8 in trace amounts. In Ben 110 MK-8(H4) and MK-6(H4) are the major menaquinones, while T1-X7T has MK-8(H4), MK-7(H4) and MK-6(H4) as its menaquinones. All three contain phosphatidylglycerol, phosphatidylglycerol and diphosphatidylglycerol as their polar lipids. These properties, together with 16S rDNA sequence data, suggest that they all belong to a single new genus for which the name *Tetrasphaera* gen. nov. is proposed. However, the lipid, cellular fatty acid profiles and DNA–DNA similarity data suggest that Ben 109T and Ben 110 are sufficiently different from T1-X7T to represent a different species of the genus *Tetrasphaera*. Strain T1-X7T represents the type species *Tetrasphaera japonica* sp. nov. of this new genus, and strains Ben 109T and Ben 110 belong to the other species, *Tetrasphaera australiensis* sp. nov.

Keywords: *Tetrasphaera*, activated sludge, G-bacteria, *Intrasporangiaceae*, biological nutrient removal

**INTRODUCTION**

The activated sludge process uses a deliberately engineered community of bacteria, protozoa and other organisms to treat domestic and industrial wastes (Seviour & Blackall, 1999; Kämpfer, 1997; Amann et al., 1995), but our understanding of the microbes present and their roles in this process is poor (Seviour & Blackall, 1999; Kämpfer, 1997; Bond et al., 1995; Amann et al., 1995). It is now clear that the application of molecular techniques and culture-in-
dependent methods (Muyzer & Smalla, 1998; Amann et al., 1998; Head et al., 1998) to the study of the microbiology of activated sludge has revealed that the diversity of microbes is much greater than once recognized using culture-dependent methods and many previously undescribed bacteria are now known to be present, often in large numbers (Seviour & Blackall, 1999; Maszenan et al., 1997, 1999a, b; Amann et al., 1995).

The limitations of relying on cell morphology and arrangement, i.e. the conventional approach to identifying the populations present are obvious in the case of the so-called 'G-bacteria'. These bacteria were first described by Cech & Hartman (1990, 1993) as Gram-negative cocci occurring in clumps and packages of tetrads. A polyphasic taxonomic approach with pure cultures of strains of these Gram-negative G-bacteria from several countries has shown that these bacteria are members of the α-Proteobacteria and represent several species of a single novel genus *Amaricoccus* (Maszenan et al., 1997). However, several quite different Gram-positive cocci have also been isolated from activated sludge plants. These include members of the genera *Micrococcus* (Painter, 1983; Wanner, 1994), *Microsphaera* (Yoshimi et al., 1996) and *Micro- lunatus* (Nakamura et al., 1995). The latter two organisms have the ability to accumulate large amounts of phosphate, but have not been reported elsewhere apart from Japan. Other populations of cocci with a similar morphology, i.e. cells in clusters or tetrads, are also found in activated sludge and are shown on culture to belong to the high-G + C group of Gram-positive bacteria. Thus Maszenan et al. (1999b) described two organisms which were novel species of *Friedmanniella* (Schumann et al., 1997), *Friedmanniella spumicola* and *Friedmanniella capsulata*, having previously isolated a coccus in tetrad arrangement which belongs to a new genus, *Tessaracoccus* (Maszenan et al., 1999a).

Many other cocci, often with the cell arrangement distinctive of the G-bacteria, have been seen in activated sludge samples (Liu et al., 1996, 1997; Carucci et al., 1995; Randall, 1994; Nielsen et al., 1999). Unfortunately, their true identities were not always confirmed as they were not cultured and characterized. Until their taxonomic status and physiology is resolved in pure culture, interpretation of their function in activated sludge systems will remain unclear.

Phylogenetic diversity among the Gram-positive cocci is well documented and it is clear that they represent a phylogenetically incoherent group of bacteria (Siefert & Fox, 1998). Such diversity will probably be reflected in physiological differences. Thus the presence of Gram-positive cocci in activated sludge samples may not always have the same significance and their functions within plants and impact on plant performance may be different. This view is supported by the observations of Liu et al. (1996) and Nakamura et al. (1995). Therefore, as part of our ongoing research to study these cocci, we have isolated further Gram-positive strains of the morphotype of the so-called G-bacteria and characterized them using a polyphasic approach. This paper reveals that they form a coherent phylogenetic group based on 16S rDNA analysis and phenotypic data. We propose that all three be placed in a novel genus *Tetrasphaera* gen. nov. However, differences in their chemotaxonomic properties and low DNA–DNA homology would suggest that they represent two different species of this novel genus. Therefore, we propose that Ben 109T and Ben 110 are given the name *Tetrasphaera australiensis* sp. nov. and strain T1-X7T is named *Tetrasphaera japonica* sp. nov.

**METHODS**

**Strain isolation and maintenance.** Strains Ben 109T and Ben 110 were isolated from activated sludge biomass from plants in Glenelg, South Australia, and the Eastern Treatment Plant, Melbourne, Victoria, Australia, respectively, by micromanipulation (Skerman, 1968) onto GS agar plates, as described by Maszenan et al. (1997). Plates were inspected microscopically to check for contamination and when colonies had developed (after 2–3 weeks), both isolates were streaked onto fresh GS plates. Purity of micromanipulated cells was confirmed microscopically and the pure cultures were stored at –80°C in GS medium plus 20% glycerol.

**Culture conditions.** The biomass for phylogenetic studies was obtained by growing strains Ben 109T and Ben 110 aerobically on GS-agar plates for 3–4 weeks at 25°C. Cells from strain T1-X7T were harvested by culturing on modified cell extract agar. The biomass for chemotaxonomic analyses was obtained by growing cultures on R2A-agar (Difco) at 25°C for 3–4 weeks. DNA for G+C determination and DNA–DNA hybridization was isolated from cells grown in liquid R-medium (Yamada & Komagata, 1972) at 28°C for 2–3 weeks.

**Microscopic studies.** Gram and polyphosphate stains were performed according to the modified Hucker method (Hucker, 1921) and the protocol of Rees et al. (1992), respectively. Gram stain results were confirmed with an alternative method employing 3% KOH (Buck, 1982). Specimens for SEM were prepared as previously described by Maszenan et al. (1997).

**Chemotaxonomic characterization.** Diaminopimelic acid isomers in hydrolysates of bacterial cells (Hasegawa et al., 1983) were determined by descending paper chromatography according to Schlüter & Kandler (1972). Cellular fatty acid methyl esters obtained by the method of Stead et al. (1992) were separated by GC using a non-polar (type OV-1) capillary column (25 m by 0.25 mm i.d.). Lipid components were analysed as described by Schumann et al. (1997). Menaquinones were analysed by reversed phase HPLC (Groth et al., 1996). Polar lipids were resolved by two-dimensional TLC on silica gel and identified by spraying...
RESULTS

Strain characteristics

A wide range of media, which included many media successfully used in the past to cultivate activated sludge bacteria, were tried in attempts to isolate the organisms from activated sludge (Seviour & Blackall, 1999). Only the GS medium of Williams & Unz (1985) was successful in supporting the growth of Ben 109T and Ben 110 after micromanipulation (Skerman, 1968). However, once in pure culture, these isolates would grow on a limited number of other media (see below). The isolates obtained fit the distinctive description of G-bacteria, i.e. cocci in pairs or arranged as tetrads or in clusters of tetrads (Fig. 1). These organisms are very slow-growing, a property shared by strain T1-X7T, which was isolated from activated sludge biomass after prolonged exposure to nutrient starvation conditions (Kataoka et al., 1996). All three took up to 3 or 4 weeks for colonies to appear on agar plates and for any turbidity to occur in liquid medium. These organisms are probably aerobic as no growth occurred down the line of inoculation in stab culture.

The growth temperature range for Ben 109T grown on GS agar was between 15 and 37 °C, while both T1-X7T and Ben 110 grew at temperatures between 20 and 37 °C. The pH range for growth of Ben 109T, Ben 110 and T1-X7T was between 6·0 and 8·0 in all cases and no growth occurred at pH 5·5 or below or at pH 8·5 and above (Table 1).

Morphological characteristics

All three organisms grew as irregular cocci in clusters of tetrads (Fig. 1c) and also sometimes as cocci in pairs (Fig. 1b). All three are Gram-positive cocci with the modified Gram staining of Hucker (1921) which was confirmed by the absence of cell stringiness with 3% KOH treatment (Buck, 1982). Flagella were never observed in any of the three isolates, showing that they are non-motile, and endospores were never observed. PolyP granules were observed in all three isolates when grown aerobically in medium incorporating glucose, acetate or propionate at an initial concentration of 0·15 g l\(^{-1}\). Temperature and pH ranges for growth, urease activity, nitrate reduction and motility were all determined as reported previously (Maszenan et al., 1997).

16S rDNA sequences and phylogenetic analysis. 16S rDNAs of Ben 109T, Ben 110 and T1-X7T were amplified and sequenced as described by Maszenan et al. (1997) and Andrews & Patel (1996). The new sequence data that were generated were aligned, an almost full length consensus 16S rRNA gene sequence assembled and checked for accuracy manually using the alignment editor ae2 (Maidak et al., 1997). If necessary, reference sequences most related to our newly generated sequence were extracted from these databases and aligned. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1450 unambiguous nucleotides were computed using DNADIST (Jukes & Cantor, 1969) and neighbour-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis and for transversion analysis (Van de Peer & De Wachter, 1993).

DNA base composition. DNA was isolated using a modification of the Marmur method (Groth et al., 1996) and was purified with Proteinase K treatment. The DNA was degraded to nucleosides using P1 nuclease and bovine intestinal mucosa alkaline phosphatase according to Mesbah et al. (1989) and the nucleosides were separated by reverse-phase HPLC (Groth et al., 1996). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to deoxythymidine.

DNA–DNA hybridization. DNA–DNA hybridization studies were carried out according to the method of De Ley et al. (1970) with the modification described by Huss et al. (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). DNA from Terracoccus luteus (DSM 44267T), Terrabacter tunescens (DSM 20308T), Intrasporangiun calvum (DSM 43043T) and Janibacter limosus (DSM 11140T) were included in this study as they were the closest phylogenetic relatives as shown in Fig. 2.

Chemotaxonomic characteristics

All three isolates are characterized by type A1\text{\textgamma} peptidoglycan with meso-diaminopimelic acid (\textit{m}-diaminopimelic acid (\textit{m}-DAP) pm) as the diagnostic amino acid (Schleifer & Kandler, 1972). The cellular fatty acid profiles of the
three strains were dominated by 14-methylpentadecanoic acid (14:1ω9c) and 14-methylhexadecanoic acid (14:1ω7c) (Table 2). However, differences were observed in strain T1-X7T which contained 1-2OH-C16:0, 2OH-C17:0, 1-C18:0, 1-5OH-C18:1 and tuberculostearic acid (TBSA) which were not found in strains Ben 109T or Ben 110. Strains Ben 109T and Ben 110 contained 12-methyltridecanoic acid (12:1ω7c) and hexadecanoic acid (16:0) which were not present in T1-X7T. These similarities and differences in cellular fatty acid composition in the three strain are summarized in Table 2. Three isoprenoid quinones, MK-8(H8), MK-6(H6) and MK-8(H8) with a composition ratio of 57:30:2 were observed in Ben 110, while Ben 109T possessed MK-8(H8), MK-8(H6) and MK-8 in the ratio of 77:6:5. T1-X7T contained, in addition to MK-8(H8), the menaquinones MK-7(H8) and MK-6(H6) in the ratio of 88:2:2 (Table 1). All three isolates possessed diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol as polar lipids. However, both Ben 109T and T1-X7T had an additional unknown phospholipid, while T1-X7T contained an unknown amino phospholipid and Ben 110 had phosphatidylethanolamine (Table 1). None of the three strains contained mycolic acids.

Physiological and biochemical characteristics

All three organisms showed a very limited ability to utilize substrates as determined using the BIOLOG GN and GP systems even after 4 weeks incubation, probably reflecting their slow growth rate and low level of metabolic activity. Alternatively, the BIOLOG systems may be unsuitable for screening these organisms. Differences in substrate utilization patterns for Ben 109T, Ben 110 and T1-X7T are summarized in Table 3. Enzymes detected in all three strains were alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase. None of these isolates produced H2S or reduced nitrate and did not hydrolyse gelatin or produce urease.

DNA base composition and DNA–DNA similarity data

DNA–DNA similarity data show that Ben 110 is related to T1-X7T, Terracoccus luteus (Präuser et al., 1997), Terrabacter tumescens (Collins et al., 1989), Janibacter limosus (Martin et al., 1997) and Intrasporangium calvum (Kalakoutski et al., 1967) with 50, 55, 41, 45 and 55% DNA homology, respectively (Table 4). The DNA base compositions of Ben 109T, Ben 110 and T1-X7T are 70, 68 and 71 mol% (Table 1), respectively.

Phylogenetic analysis

An almost complete sequence of the 16S rRNA gene for strains Ben 109T (1470 nt), Ben 110 (1477 nt), T1-X7T (1445 nt) corresponding to positions 21–1513,
13–1512 and 15–1478 of the Escherichia coli sequence according to the nomenclature of Winker & Woese (1991), respectively, was obtained. Phylogeny after 16S rDNA analysis indicates all three isolates belong to the high-G+C group of the Gram-positive bacteria in the domain Bacteria. The phylogenetic tree shows that Ben 109T, Ben 110 and T1-X7T form a cluster at a level of 97-0% similarity, with Terrabacter, Terracoccus, Sanguibacter (Fernández-Garayzabal et al., 1995; Pascual et al., 1996), Intrasporangiium and Janibacter as their closest relatives (Fig. 2). Transversion analysis did not change the relative positions of the newly isolated strains in the phylogenetic tree. These organisms are currently classified in the Intrasporangiaceae family in the suborder Micrococcineae (Stackebrandt et al., 1997), even though the genera Terracoccus and Janibacter were not included in the proposed scheme of Stackebrandt et al. (1997).

Table 1. Comparative phenotypic properties of the genera Janibacter, Terrabacter, Terracoccus, Intrasporangiium, Sanguibacter and strains Ben 109T, Ben 110 and T1-X7T

<table>
<thead>
<tr>
<th>Phenotypic property</th>
<th>Ben 109T</th>
<th>Ben 110</th>
<th>T1-X7T</th>
<th>Janibacter*</th>
<th>Terrabacter†</th>
<th>Terracoccus‡</th>
<th>Intrasporangiium§</th>
<th>Sanguibacter‖</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O₂ requirement</strong></td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Obligately aerobic</td>
<td>Strictly aerobic</td>
<td>Aerobic</td>
<td>Facultatively anaerobic</td>
</tr>
<tr>
<td><strong>Cell morphology</strong></td>
<td>Coccis, single, in pairs, in tetrad arrangement and clusters (0.5–1.0 µm)</td>
<td>Coccis, single, in pairs, in tetrad arrangement and clusters (0.4–1.1 µm)</td>
<td>Coccis, single, in pairs, in tetrad arrangement and clusters (0.6–1.4 µm)</td>
<td>Coccis, single, in pairs, in tetrad and small clusters (0.9–1.3 µm)</td>
<td>Rods and rods in tetrads and small clusters (0.6–1.0 x 2.0–7.0 µm)</td>
<td>Coccis, in pairs, tetrads and small clusters (0.7–1.3 µm)</td>
<td>Soil and at water interface of duck pond</td>
<td>Soil and at water/soil interface of duck pond</td>
</tr>
<tr>
<td><strong>Habitat</strong></td>
<td>Sewage treatment plant in South Australia</td>
<td>Sewage treatment plant in Melbourne, Victoria, Australia</td>
<td>Activated sludge biomass in Japan</td>
<td>Sludge, sewage waste</td>
<td>Sludge, sewage</td>
<td>Soil</td>
<td>From air in school dining room</td>
<td>Blood and milk of healthy cows</td>
</tr>
<tr>
<td><strong>Optimum growth pH</strong></td>
<td>7.0</td>
<td>7.0–7.5</td>
<td>7.0</td>
<td>ND</td>
<td>ND</td>
<td>6.0–7.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Growth pH range</strong></td>
<td>6.0–8.0</td>
<td>6.0–8.0</td>
<td>6.0–8.0</td>
<td>ND</td>
<td>ND</td>
<td>4.5–9.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Catalase</strong></td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td><strong>Oxidase</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td><strong>Nitrate reduction</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>G+C content (mol%)</strong></td>
<td>70</td>
<td>68</td>
<td>71</td>
<td>70</td>
<td>70–73</td>
<td>73</td>
<td>68</td>
<td>69–70</td>
</tr>
<tr>
<td><strong>Production of H₂S</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Production of indole</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
</tr>
<tr>
<td><strong>Major menaquinone</strong></td>
<td>MK-8(H₁)</td>
<td>MK-8(H₁), MK-8(H₁), MK-8(H₁)</td>
<td>MK-8(H₁)</td>
<td>MK-8(H₁)</td>
<td>MK-8(H₁)</td>
<td>MK-8(H₁)</td>
<td>MK-8</td>
<td>MK-8</td>
</tr>
<tr>
<td><strong>Spore formation</strong></td>
<td>No spore formation</td>
<td>No spore formation</td>
<td>No spore formation</td>
<td>No spore formation</td>
<td>No spore formation</td>
<td>No spore formation</td>
<td>Spore formation</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Diaminopimelic acid/murein type</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>t-Lysine (A4γ)</td>
</tr>
<tr>
<td><strong>Gelatin hydrolysis</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Data from Martin et al. (1997).
† Data from Collins et al. (1989).
‡ Data from Prauser et al. (1997).
§ Data from Kalakoutsii et al. (1967) and Kalakoutsii (1989).
‖ Data from Fernández-Garayzabal et al. (1995) and Pascual et al. (1996).
DISCUSSION

All three isolates described here, Ben 109\textsuperscript{T}, Ben 110 and T1-X7\textsuperscript{T}, from activated sludge are aerobic, non-motile, non-spore-forming, very slow-growing, Gram-positive cocci, occurring singly and in pairs, but predominantly as tetrads and clusters. All can store phosphate as polyphosphate granules. Our understanding of the bacteria responsible for phosphate removal in activated sludge is poor, although some work has shown that bacteria possessing menaquinone MK-8(H\textsubscript{2}) are a major component of communities of such systems (Hiraishi et al., 1998). All three bacteria in this study contain MK-8(H\textsubscript{2}), as does Janibacter which was also isolated from activated sludge (Martin et al., 1997). Therefore, genus-specific probes for their in situ detection, together with microautoradiography (Andreasen & Nielsen, 1997; Nielsen et al., 1998) would be useful to clarify their contribution, if any, to this process.

Table 2. Percentage cellular fatty acid composition of Ben 109\textsuperscript{T}, Ben 110, T1-X7\textsuperscript{T}, Terracoccus luteus, Terrabacter tumescens, Janibacter limosus, Sanguibacter and Intrasporangium calvum

| Fatty acid | Ben 109\textsuperscript{T} | Ben 110 | T1-X7\textsuperscript{T} | Terracoccus luteus\* | Terrabacter tumescens\* | Janibacter limosus\* | Sanguibacter ST-26\§ | Sanguibacter ST-86\¶ | Sanguibacter ST-74\¶ | Sanguibacter ST-167\¶ | Intrasporangium calvum\¶ |
|------------|-----------------|---------|------------------|-------------------|-------------------|-------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| i-C\textsubscript{16:0} | – | – | – | 1-1 | – | – | – | – | – | – | – | – |
| ai-C\textsubscript{16:0} | – | – | – | 1 | – | – | – | – | – | – | – | – |
| i-C\textsubscript{17:0} | 26 | 15 | – | 3-5 | 242 | 05 | 15 | – | 1-1 | 0-9 | 5-0 | – |
| C\textsubscript{18:0} | – | – | – | 18 | – | 04 | 148 | 13-1 | 5-8 | 4-1 | – | – |
| i-C\textsubscript{18:0} | 121 | 93 | 64 | 34-3 | 35-4 | 1-7 | 36 | 3-7 | 3-2 | 4-4 | 3-5 | – |
| C\textsubscript{18:1} \(\Delta\textsubscript{6}\) | – | – | – | 15 | – | – | – | – | – | – | – | – |
| ai-C\textsubscript{18:1} | 76 | 14 | 30 | 30-4 | 3-7 | 03 | 389 | 42-1 | 11-4 | 22-7 | 12-6 | – |
| C\textsubscript{19:0} | – | – | – | – | – | – | 10 | 1-8 | 1-6 | 1-3 | – | – |
| i-C\textsubscript{19:0} | 49 | 13 | 1-1 | 1-9 | 1-1 | 58 | 1-1 | – | 0-5 | – | 2-7 | – |
| C\textsubscript{19:1} \(\Delta\textsubscript{6}\) | – | 12 | – | 0-1 | – | 0-1 | – | – | – | – | 1-5 | – |
| i-C\textsubscript{19:1} | 271 | 348 | 258 | 25 | 13-4 | 17-5 | 27 | 2-5 | 5-4 | 3-4 | 12-3 | – |
| C\textsubscript{20:0} | 59 | 1-7 | 0-0 | 0-9 | 1-4 | 2-4 | – | – | – | – | – | – |
| i-C\textsubscript{20:0} | 8-4 | 6-2 | 12-4 \& 4-9 | 0-9 | – | – | – | – | – | – | – | – |
| C\textsubscript{20:1} \(\Delta\textsubscript{9}\) | – | 20 | 2-2 | 1-0 | – | 1-3 | – | – | 1-4 | 2-3 | 1-7 | – |
| ai-C\textsubscript{20:1} | 141 | 10-1 | 20-5 | 4-1 | – | – | 1-2 | 2-0 | 2-4 | 6-3 | 3-9 | – |
| C\textsubscript{21:0} | – | 12 | 1-3 | 1-4 | 20-5 | – | 0-5 | – | – | – | 3-7 | – |
| i-C\textsubscript{21:0} | – | – | – | – | – | – | 24 | 2-9 | – | – | – | – |
| ai-C\textsubscript{21:0} | 4-7 | 22 | 6-9 | – | – | 0-2 | – | – | – | – | – | – |
| 2OH-C\textsubscript{19:0} | – | 6-1 | – | – | – | – | – | – | – | – | – | – |
| 2OH-i-C\textsubscript{18:0} | – | – | 5-3 | – | – | – | – | – | – | – | – | – |
| i-C\textsubscript{19:1} \(\Delta\textsubscript{9}\) | – | – | 13 | – | 0-7 | – | – | – | – | – | – | – |
| C\textsubscript{21:0} | 3-7 | 97 | 2-2 | 3-6 | – | 8-1 | – | – | – | 2-2 | 6-3 | – |
| i-C\textsubscript{21:1} | – | – | – | – | – | – | – | – | – | – | – | – |
| TBSA | – | – | – | 1-5 | – | – | – | – | – | – | – | – |

* Data from Prauser et al. (1997).
† Data from Prauser et al. (1997).
‡ Data from Andreasen et al. (1998).
§ Data from Kalakoutsik et al. (1967); Kalakoutsik (1989), Schumann et al. (1997).
¶ Two isomers of C\textsubscript{16:1}.

Table 3. Comparative substrate utilization patterns of strains Ben 109\textsuperscript{T}, Ben 110 and T1-X7\textsuperscript{T}

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>T1-X7\textsuperscript{T}</th>
<th>Ben 109\textsuperscript{T}</th>
<th>Ben 110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 40</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(\beta)-Trehalose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Methyl glucose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(\alpha)-Xylose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adenosine-5'-monophosphate</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lysine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>(\beta)-Galactosidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Determined by BIOLOG GN and GP systems, Microbact 24E and API ZYM. None of the other substrates (a total 155) gave a positive result even after several weeks of incubation.
Sanguibacter relatives are the genera Terrabacter discussed below, appear now to represent a more Intrasporangiaceae family in the domain Actinobacteria cluster at a high similarity value (50%).

Phylogenetically, 16S rDNA sequence analyses reveal that Ben 109T, Ben 110 and T1-X7T form a coherent cluster at a high similarity value (>97%) in the class Actinobacteria in the domain Bacteria. Their closest relatives are the genera Terrabacter, Terracoccus, Sanguibacter, Intrasporangium and Janibacter, all in the family Intrasporangiaceae (Fig. 2), which, as discussed below, appears now to represent a more heterogeneous group of bacteria than once thought.

Table 4. Levels of DNA–DNA similarity between strains Ben 109T, Ben 110, T1-X7T, Terracoccus luteus, Terrabacter tumescens, Janibacter limosus and Intrasporangium calvum

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage relatedness to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ben 109T</td>
</tr>
<tr>
<td>Ben 109T</td>
<td>89</td>
</tr>
<tr>
<td>Ben 110</td>
<td>41</td>
</tr>
<tr>
<td>T1-X7T</td>
<td></td>
</tr>
<tr>
<td>Terracoccus luteus</td>
<td>ND</td>
</tr>
<tr>
<td>Terrabacter tumescens</td>
<td>ND</td>
</tr>
<tr>
<td>Janibacter limosus</td>
<td>40</td>
</tr>
<tr>
<td>Intrasporangium calvum</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

All three strains have m-A4pm and type A1γ peptidoglycan, a feature they share with members of the genus Janibacter, but none of the other genera mentioned, which all possess t1-A9PM as their dibasic amino acid and type A2γ peptidoglycan instead (Table 1). However, cellular fatty acid profiles, together with DNA homology data and 16S rDNA sequences signature positions (Table 5) suggest that Ben 109T, Ben 110 and strain T1-X7T represent members of novel single genus, which we have called Tetrasphaera gen. nov., and should not be placed in any of the genera Terrabacter, Terracoccus, Sanguibacter, Intrasporangium or Janibacter.

Even though their 16S rDNA sequences support the view that these isolates all belong to the same genus, in our opinion these and other characters are not consistent with the idea that all three represent a single species. Thus, although a DNA–DNA homology value of >70% shows Ben 109T and Ben 110 to be members of the same genomic species (Stackebrandt & Goebel, 1994; Fox et al., 1992), this value between T1-X7T and both Ben 109T and Ben 110 is <50% (Table 4) and illustrates that the former is not as closely related to these strains as they are to each other. In addition, the presence of menaquinone MK-8(H4) and fatty acid i-C14:0 in both Ben 109T and Ben 110, but not in T1-X7T, which has MK-7(H4) and hydroxylated fatty acids, i-C18:0 and i-C18:1γ, and other phenotypic differences

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**Fig. 2.** Dendrogram based on 16S rDNA sequence data indicating the positions of *Tetrasphaera australiensis* (Ben 109T and Ben 110) and *Tetrasphaera japonica* (T1-X7T) within the family Intrasporangiaceae (shaded box), suborder Micrococcineae and their relationship to other members of the order Actinomycetales. All sequences used in the analysis were obtained from the Ribosomal Database Project, version 7.0 (Maidak et al., 1997) with the exception of *Sanguibacter inulinus*, *Sanguibacter keddieii*, *Sanguibacter suarezii*, *Intrasporangium calvum*, *Janibacter limosus* and *Terracoccus luteus*, which were extracted from GenBank (accession nos X79451, X79450, X79452, D85486, Y08539 and Y11928, respectively). Bootstrap values greater than 90% (generated from 100 data sets) are shown at the branching points. The two triangles represent clusters consisting of *Sanguibacter inulinus*, *Sanguibacter keddieii*, *Sanguibacter suarezii* (designated Sanguibacter group) and the genera Propionibacterium, Luteococcus, Propioniferax, Microlunatus and Tessaracoccus (designated Propionibacteriaceae). Scale bar indicates 2 nt substitutions per 100 nt.
### Table 5. 16S rDNA nucleotide signatures for Ben 109T, Ben 110, T1-X7T and members of the family Intrasporangiaceae

<table>
<thead>
<tr>
<th>Position*</th>
<th>Intrasporangiaceae</th>
<th>Ben 109T</th>
<th>Ben 110</th>
<th>T1-X7T</th>
<th>Intrasporangium calvum</th>
<th>Sanguibacter</th>
<th>Terrabacter</th>
<th>Janibacter</th>
<th>Terracoccus</th>
</tr>
</thead>
<tbody>
<tr>
<td>30–553</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
</tr>
<tr>
<td>630</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>694</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A†‡ §</td>
<td></td>
</tr>
<tr>
<td>838–848</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
</tr>
<tr>
<td>859</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>1007–1022</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
</tr>
<tr>
<td>1134–1140</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>A-G</td>
</tr>
</tbody>
</table>

* E. coli numbering.
† Terrabacter sp. DDE-1 (GenBank U96645) has A-T instead of G-C at position 140–223, T-G instead of G-C at position 157–164, A instead G at position 694 and C-T instead of C-G at position 1007–1022.
‡ Terrabacter sp. DPO 1361 (EMBL Y08853) has G-T instead G-C at position 140–223, T-G instead of G-C at position 157–164, A instead G at position 694 and C-T instead of C-G at position 1007–1022.
¶ Sanguibacter keddieii strain NCFB 3025 (EMBL X79450) has C-T instead of C-G at position 1007–1022.
* Terracoccus luteus strain DSM 44267 (EMBL Y11928) has A-G instead of C-G at position 1134-1140.

### Table 6. 16S rDNA nucleotide signatures for Ben 109T, Ben 110, T1-X7T and members of the family Intrasporangiaceae within the suborder Micrococcineae (Stackebrandt et al., 1997)

<table>
<thead>
<tr>
<th>Position*</th>
<th>Micrococcineae</th>
<th>Ben 109T</th>
<th>Ben 110</th>
<th>T1-X7T</th>
<th>Terrabacter†</th>
<th>Intrasporangium calvum</th>
<th>Sanguibacter‡</th>
<th>Janibacter§</th>
<th>Terracoccus luteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>449</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>600–638</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
<td>T-G</td>
</tr>
<tr>
<td>952–1229</td>
<td>C-G</td>
<td>T-A</td>
<td>T-A</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
<td>C-G</td>
</tr>
</tbody>
</table>

* E. coli numbering.
† Terrabacter tumescens (GenBank AF005023), Terrabacter sp. DDE-1 (GenBank U96645) and Terrabacter sp. DPO 1361 (EMBL Y08853) were used for comparison.
‡ Sanguibacter inulinus strain ST-50 (EMBL X79452), Sanguibacter keddieii strain ST-74 (EMBL X79450), Sanguibacter sp. (EMBL Y09657) and Sanguibacter suarezii strain ST-26 (EMBL X79451) were used for comparison.
§ Janibacter limosus strain DSM 11141 (EMBL Y08539) and Janibacter limosus strain DSM 11141 (EMBL Y08540) were used for comparison.
suggest that strain T1-X7{T} is a representative of another species within the genus Tetrasphaera. This
taxonomic proposition is given further support when the
16S rDNA signature positions are considered
(Table 5). Thus both Ben 109{T} and Ben 110 possess T-
A instead of G-C at position 157–164 as also found in
T1-X7{T}, A-T instead of G-T at 658–748 and G-C
instead of A-T at 1133–1141. Hence, we propose that
Ben 109{T} and Ben 110 be placed in the same species in
the genus Tetrasphaera as Tetrasphaera australiensis
sp. nov. and strain T1-X7{T} as Tetrasphaera japonica
sp. nov.

It is clear, using the taxonomic scheme of Stackebrandt
et al. (1997) for Actinobacteria, based solely on 16S
rDNA signatures that Tetrasphaera fits readily within
the suborder Micrococccinae, even though its
signatures do not precisely match those for this group.
Thus T-A replaces C-G at position 952–1229. All the
other closest related genera (Table 6) show complete
agreement with the scheme of Stackebrandt et al.
(1997) for this suborder.

However, Tetrasphaera does have multiple mismatches
(Table 5) with the signatures considered diagnostic for
the family Intrasporangiaceae by Stackebrandt et al.
(1997), although Tetrasphaera japonica shows closer
agreement (Tables 5 and 6) than Tetrasphaera australiensis
two mismatches compared to four mismatches).
Furthermore, Janibacter (Martin et al., 1997), Terracoccus
(Prauser et al., 1997) and Sanguibacter (Pascal et al., 1996)
each has several mismatches in their signature for the current
delineation of the family Intrasporangiaceae (Tables 5
and 6). It is probable that the current classification of
this group of bacteria will need modification when more
isolates of closely related members of the Actino-
bacteria are obtained and characterized, and as such
should be viewed as preliminary. The recent description
of Tesseracoccus (Maszenan et al., 1999a), which
also shows considerable departures from this proposal,
reinforces this view.

Description of Tetrasphaera gen. nov.

Tetrasphaera (Te.tra.sphae'ra. Gr. n. tetra four; M.L.
fem. n. sphaera sphere; M.L. fem. n. Tetrasphaera,
four spherical bacterial cells).

Originates from activated sludge. Cells are Gram-
positive, aerobic and coccoid in shape (0.5–1.4 μm
diam.) occurring singly or in pairs but predominantly
as tetrads and in clusters. Cells are non-motile and
non-spor-forming. Cultures grow very slowly and
utilize a limited number of substrates. Catalase-
positive, urease-negative and able to store poly-
phosphate. Do not produce H₂S or indole. Cannot
hydrolyse gelatin, but can utilize propionate, acetate,
glucose and arbutin. Cell wall peptidoglycan contains
meso-diaminopimelic acid (m-A,p,pm) which charac-
terizes type A1γ murein. The major menaquinone is
MK-8(H₄), Mycolic acids are absent. The pH range
supporting growth is between 6.0 and 8.0. The main
cellular fatty acid is iso-C₁₆:₀ and ai-C₁₇:₀ i-C₁₈:₁ i-
C₁₅:₀ as well as C₁₆:₁ are present in smaller amounts.
All three have G-C at position 1133–1141 and T-A at
position 952–1229. The G+C content of the DNA is
between 68 and 71%. Phylogenetically, its closest
relatives are Terrabacter, Terracoccus, Sanguibacter,
Intrasporangium and Janibacter, all in the family
Intrasporangiaceae, suborder Micrococccinae. The
type species is Tetrasphaera japonica.

Description of Tetrasphaera japonica sp. nov.

Tetrasphaera japonica (ja.po'ni.ca. N.L. adj. japonica
pertaining to Japan, from where the isolate originated).
The morphology, chemotaxonomic properties and
phenotypic characteristics are the same as those
already described for the genus. This species is weakly
catalase-positive. It possesses the respiratory quinones
MK-7(H₄) and MK-6(H₄) in addition to the major
menaquinone MK8(H₄) and contains an unknown
amino phospholipid. It also possesses i-2OH-C₁₆:₀ 2-
OH-C₁₇:₀ i-C₁₈:₀ i-C₁₉:₁ and tuberculosae acid
(TBSA) and lacks both iso-C₁₄:₀ and C₁₆:₀. It can
utilize pyruvic acid. The G+C content is 71 mol %. It
has G-C at position 1133–1141 and G-C at position
1134–1140 of its 16S rDNA. The type strain, T1-X7{T},
has been deposited in the Australian Collection of
Microorganisms, University of Queensland, Brisbane,
Australia, as strain ACM 5116{T}.

Description of Tetrasphaera australiensis sp. nov.

Tetrasphaera australiensis (aus.tral.ie.nis. L. nom.
fem. adj. australiensis of Australia, from where the
isolates originated).

Two strains fit the description of this species based on
phylogenetic analysis and phenotypic properties. Both
Ben 109{T} and Ben 110 contain MK-8(H₄) as the major
menaquinone and MK-8(H₄) is present in trace
amounts. In addition, strain Ben 110 possesses a large
amount of MK-6(H₄) while Ben 109{T} has MK-8 in
trace amounts instead. They can both utilize Tween 40,
Tween 80, sucrose, D-trehalose, 3-methylglucose,
d-xylose and adenosine-5'-monophosphate, and have T-
A at position 157–164, A-T at position 658–748 and A
at position 694 of their 16S rDNA. Their G+C content
is 68–70 mol %. The type strain, Ben 109{T}, has
been deposited in the Australian Collection of
Microorganisms, University of Queensland, Brisbane,
Australia, as strain ACM 5117{T}, while strain (Ben 110)
is deposited as strain ACM 5118.

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Pimelobacter jensenii comb. nov.

Enhanced biological phosphate removal systems. Water Res on 2,4-diaminobutyric acid.


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activated sludge biomass, and descriptions of *Amaricoccus veronensis* sp. nov., *Amaricoccus tamworthiensis* sp. nov., *Amaricoccus macauensis* sp. nov. and *Amaricoccus kapilicensis* sp. nov. *Int J Syst Bacteriol* 49, 727–734.


