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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 109^T 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-X7^T 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 109^T, Ben 110 and T1-X7^T is 14-methylpentadecanoic acid (i-$C_{16:0}$). The major menaquinones of Ben 109^T are MK-8(H_4), with MK-8(H_2) and MK-8 in trace amounts. In Ben 110 MK-8(H_4) and MK-6(H_4) are the major menaquinones, while T1-X7^T has MK-8(H_4), MK-7(H_4) and MK-6(H_4) as its menaquinones. All three contain phosphatidylinositol, 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 109^T and Ben 110 are sufficiently different from T1-X7^T to represent a different species of the genus *Tetrasphaera*. Strain T1-X7^T represents the type species *Tetrasphaera japonica* sp. nov. of this new genus, and strains Ben 109^T 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-

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The GenBank accession numbers for the 16S rRNA gene sequences of Ben 109^T, Ben 110 and T1-X7^T are AF125091, AF125090 and AF125092, respectively.

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 *Microlunatus* (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 109^T and Ben 110 are given the name *Tetrasphaera australiensis* sp. nov. and strain T1-X7^T is named *Tetrasphaera japonica* sp. nov.

METHODS

Strain isolation and maintenance. Strains Ben 109^T 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. Strain T1-X7^T was the isolate described by Kataoka *et al*. (1996). It was grown on modified cell extract agar which incorporated 0·25% Casamino acids instead of the cell extract from activated sludge described in the original paper (Kataoka *et al*., 1996). Strain T1-X7^T was stored in cell extract medium with 20% glycerol at −80 °C

Culture conditions. The biomass for phylogenetic studies was obtained by growing strains Ben 109^T and Ben 110 aerobically on GS agar plates for 3–4 weeks at 25 °C. Cells from strain T1-X7^T 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 Schleifer & 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

with specific reagents (Collins & Jones, 1980). Absence of mycolic acids was demonstrated by TLC (Minnikin *et al.*, 1975).

Biochemical and physiological characterization. Enzymic profiles and biochemical characteristics of Ben 109^T, Ben 110 and T1-X7^T were determined using the API ZYM (BioMérieux) and Microbact 24E systems (Oxoid) as described previously (Maszenan *et al.*, 1997). A screening of carbon substrates utilized was performed with the BIOLOG GP and GN (Special Diagnostics) microplate systems. Oxidase and catalase activities were determined using standard methods (Smibert & Krieg, 1994). Storage polymer granule detection was by microscopic examination of cells stained for polyphosphate (PolyP) and polyhydroxyalkanoate (PHA) (Rees *et al.*, 1992) which were grown in GS 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 109^T, Ben 110 and T1-X7^T 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). These were compared with other sequences in the GenBank database (Benson *et al.*, 1993) using BLAST (Altschul *et al.*, 1997), and in the Ribosomal Database Project, version 7.0 using SIMILARITY_RANK and SUGGEST_TREE (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 44267^T), *Terrabacter tumescens* (DSM 20308^T), *Intrasporangium calvum* (DSM 43043^T) and *Janibacter limosus* (DSM 11140^T) were included in this study as they were the closest phylogenetic relatives as shown in Fig. 2.

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 109^T 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-X7^T, 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 109^T grown on GS agar was between 15 and 37 °C, while both T1-X7^T and Ben 110 grew at temperatures between 20 and 37 °C. The pH range for growth of Ben 109^T, Ben 110 and T1-X7^T 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 as sole carbon source. However no polyhydroxyalkanoate (PHA) granules were detected. The mean cell diameters of individual coccal cells were 0·5–1·0 μm for Ben 109^T, 0·4–1·1 μm for Ben 110 and 0·6–1·4 μm for T1-X7^T grown on GS agar and modified cell extract agar, respectively.

Chemotaxonomic characteristics

All three isolates are characterized by type A1γ peptidoglycan with *meso*-diaminopimelic acid (*m*-A_2pm) as the diagnostic diamino acid (Schleifer & Kandler, 1972). The cellular fatty acid profiles of the

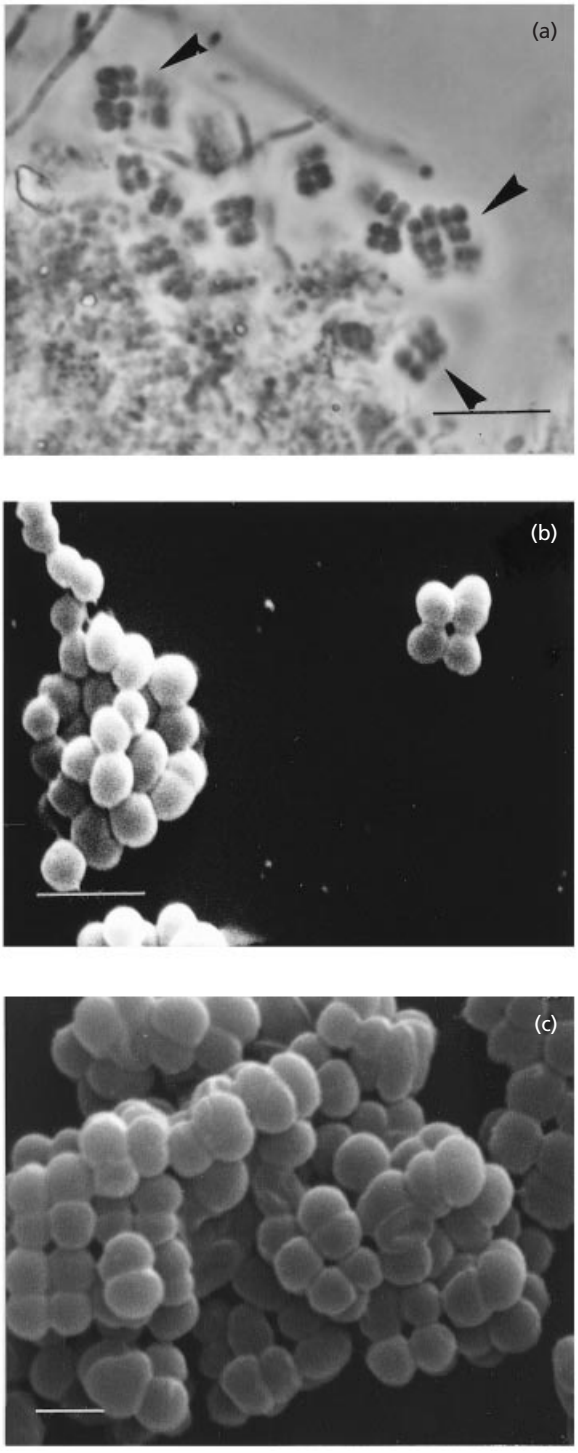

Fig. 1. (a) Tetrads of Gram-positive G-bacteria (arrows) in a mixed liquor sample from the Glenelg Plant, South Australia. Bar, 10 μm. (b) Scanning electron micrograph of a pure culture of Ben 110 from the Eastern Treatment Plant, Melbourne, Australia, showing a tetrad and aggregates of coccoid cells. Bar, 2 μm. (c) Scanning electron micrograph of an axenic culture of Ben 109^T from the Glenelg Plant, South Australia, showing coccoid cells in tetrads organized into clusters. Bar, 1 μm.

three strains were dominated by 14-methylpentadecanoic acid (i-$C_{16:0}$) and 14-methylhexadecanoic acid (ai-$C_{17:0}$) (Table 2). However, differences were observed in strain T1-X7^T which contained i-2OH-$C_{16:0}$, 2OH-$C_{17:0}$, i-$C_{18:0}$, i-$C_{18:1}$ and tuberculostearic acid (TBSA) which were not found in strains Ben 109^T or Ben 110. Strains Ben 109^T and Ben 110 contained 12-methyltridecanoic acid (i-$C_{14:0}$) and hexadecanoic acid ($C_{16:0}$) which were not present in T1-X7^T. These similarities and differences in cellular fatty acid composition in the three strain are summarized in Table 2. Three isoprenoid quinones, MK-8(H_4), MK-6(H_4) and MK-8(H_2) with a composition ratio of 57:30:2 were observed in Ben 110, while Ben 109^T possessed MK-8(H_4), MK-8(H_2) and MK-8 in the ratio of 77:6:5. T1-X7^T contained, in addition to MK-8(H_4), the menaquinones MK-7(H_4) and MK-6(H_4) in the ratio of 88:2:2 (Table 1). All three isolates possessed diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol as polar lipids. However, both Ben 109^T and T1-X7^T had an additional unknown phospholipid, while T1-X7^T 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 109^T, Ben 110 and T1-X7^T 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 H_2S 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-X7^T, *Terracoccus luteus* (Prauser *et al.*, 1997), *Terrabacter tumescens* (Collins *et al.*, 1989), *Janibacter limosus* (Martin *et al.*, 1997) and *Intrasporangium calvum* (Kalakoutskii *et al.*, 1967) with 50, 55, 41, 45 and 55% DNA homology, respectively (Table 4). The DNA base compositions of Ben 109^T, Ben 110 and T1-X7^T are 70, 68 and 71 mol% (Table 1), respectively.

Phylogenetic analysis

An almost complete sequence of the 16S rRNA gene for strains Ben 109^T (1470 nt), Ben 110 (1477 nt), T1-X7^T (1445 nt) corresponding to positions 21–1513,

Table 1. Comparative phenotypic properties of the genera *Janibacter*, *Terrabacter*, *Terracoccus*, *Intrasporangium*, *Sanguibacter* and strains Ben 109^T, Ben 110 and T1-X7^T

+, Positive result; −, negative result; ND, not determined. An arrow indicates a growth cycle, i.e. cocci to rods in *Janibacter*, rods to cocci in *Terrabacter*. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine, PL, unknown phospholipid(s); PIM, phosphatidyl inositol mannosides; APL, unknown amino phospholipid.

Phenotypic property	Ben 109^T	Ben 110	T1-X7^T	*Janibacter**	*Terrabacter*†	*Terracoccus*‡	*Intrasporangium*§	*Sanguibacter*‖
O_2 requirement	Aerobic	Aerobic	Aerobic	Aerobic	Obligately aerobic	Strictly aerobic	Aerobic	Facultatively anaerobic
Cell morphology	Cocci, single, in pairs, in tetrad arrangement and clusters (0·5–1·0 μm)	Cocci, single, in pairs, in tetrad arrangement and clusters (0·4–1·1 μm)	Cocci, single, in pairs, in tetrad arrangement and clusters (0·6–1·4 μm)	Cocci → rods, single, in pairs and also in irregular clumps (0·3–1·2 μm)	Rods ← cocci (0·6–1·0 × 2·0–7·0 μm)	Cocci, in pairs, tetrads and small clusters (0·7–1·3 μm)	Sporangia	Short irregular rods
Habitat	Sewage treatment plant in South Australia	Sewage treatment plant in Melbourne, Victoria, Australia	Activated sludge biomass in Japan	Sludge, sewage waste	Soil	Soil and at water/soil interface of duck pond	From air in school dining room	Blood and milk of healthy cows
Optimum growth temperature (°C)	25	25	25	28	25–30	28	37	25–30
Growth temperature range (°C)	15–37	20–37	20–37	28–37	10–35	15–37	28–37	25–30
Optimum growth pH	7·0	7·0–7·5	7·0	ND	ND	6·0–7·2	ND	ND
Growth pH range	6·0–8·0	6·0–8·0	6·0–8·0	ND	ND	4·5–9·5	ND	ND
Catalase	+	+	±	+	+	+	ND	+
Oxidase	+	−	+	−	−	−	ND	−
Nitrate reduction	−	−	−	+	+	−	+	+
Urease	−	−	−	−	−	+	ND	−
G+C content (mol%)	70	68	71	70	70–73	73	68	69–70
Production of H_2S	−	−	−	+	ND	+	ND	ND
Production of indole	−	−	−	+	ND	ND	ND	ND
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Rods are non-motile, occasionally motile	Non-motile	Non-motile	Non-motile
Major menaquinone	MK-8(H_4)	MK-8(H_4), MK-6(H_4)	MK-8(H_4)	MK-8(H_4)	MK-8(II, III-H_4)	MK-8(H_4)	MK-8	MK-9(H_4)
Spore formation	No spore formation	No spore formation	No spore formation	No spore formation	No spore formation	No spore formation	Spore formation	ND
Diamino acid/murein type	*m*-A_2pm (A1γ)	*m*-A_2pm (A1γ)	*m*-A_2pm (A1γ)	*m*-A_2pm (A1γ)	LL-A_2pm (A3γ)	LL-A_2pm (A3γ)	LL-A_2pm (A3γ)	L-Lysine (A4α)
Gelatin hydrolysis	−	−	−	+	+	+	−	+
Polar lipids	DPG, PG, PI, PL	DPG, PG, PI, PE	DPG, PG, PI, PL, APL	DPG, PG, PI	DPG, PI, PE, PL	DPG, PG, PI, PE	DPG, PG, PI, PIM	ND

* Data from Martin *et al.* (1997).

† Data from Collins *et al.* (1989).

‡ Data from Prauser *et al.* (1997).

§ Data from Kalakoutskii *et al.* (1967) and Kalakoutskii (1989).

‖ Data from Fernández-Garayzábal *et al.* (1995) and Pascual *et al.* (1996).

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 109^T, Ben 110 and T1-X7^T form a cluster at a level of 97·0% similarity, with *Terrabacter*, *Terracoccus*, *Sanguibacter* (Fernández-Garayzábal *et al.*, 1995; Pascual *et al.*, 1996), *Intrasporangium* 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 2. Percentage cellular fatty acid composition of Ben 109^T, Ben 110, T1-X7^T, *Terracoccus luteus*, *Terrabacter tumescens*, *Janibacter limosus*, *Sanguibacter* and *Intrasporangium calvum*

Values less than 1% are not shown. Abbreviations for fatty acids are as follows: $C_{16:0}$, hexadecanoic acid; $C_{18:1}$, octadecenoic acid; i-$C_{15:0}$,13-methyltetradecanoic acid; ai-$C_{15:0}$, 12-methyltetradecanoic acid; TBSA, tuberculostearic acid. i, iso; ai, anteiso.

Fatty acid	Ben 109^T	Ben 110	T1-X7^T	*Terracoccus luteus**	*Terrabacter tumescens*†	*Janibacter limosus*‡	*Sanguibacter* ST-26§	*Sanguibacter* ST-86§	*Sanguibacter* ST-74§	*Sanguibacter* ST-167§	*Intrasporangium calvum*‖
i-$C_{13:0}$	–	–	–	1·1	–	–	–	–	–	–	–
ai-$C_{13:0}$	–	–	–	1·1	–	–	0·8	–	–	–	–
i-$C_{14:0}$	2·6	1·5	–	3·5	24·2	0·5	1·5	–	1·1	0·9	5·0
$C_{14:0}$	–	–	–	1·8	–	0·4	14·8	13·1	5·8	4·1	–
i-$C_{15:0}$	12·1	9·3	6·4	34·3	35·4	1·7	3·6	3·7	3·2	4·4	37·8
i-$C_{15:1}$	–	–	–	–	1·5	–	–	–	–	–	3·5
ai-$C_{15:0}$	7·6	1·4	3·0	30·4	3·7	0·3	38·9	42·1	11·4	22·7	12·6
ai-$C_{15:1}$	–	–	–	–	–	–	1·0	1·8	1·6	1·3	
$C_{15:0}$	4·0	1·3	1·1	1·9	1·1	3·8	1·1	–	0·5	–	2·7
$C_{15:1}$	–	1·2	–	0·1	–	0·3	–	–	–	–	1·5
i-$C_{16:0}$	27·1	34·8	25·8	2·5	13·4	17·5	2·7	2·5	5·4	3·4	12·3
i-$C_{16:1}$	9·7	12·4	3·2	–	8·6	0	–	–	–	–	3·1
$C_{16:0}$	5·9	1·7	–	10·0	–	3·9	33·7	34·8	53·3	34·5	1·2
$C_{16:1}$	8·4	6·2	1·2 & 4·4¶	0·9	1·4	2·4	–	–	–	–	–
i-$C_{17:0}$	–	2·0	2·2	1·0	–	1·3	–	–	1·4	2·3	1·7
ai-$C_{17:0}$	14·1	10·1	20·5	4·1	–	–	1·2	2·0	2·4	6·3	3·9
i-$C_{17:1}$	–	2·0	2·3	–	–	0	–	–	–	–	3·7
$C_{17:0}$	–	1·2	1·3	1·4	–	20·5	–	–	0·5	–	1·0
$C_{17:1}$	–	–	–	–	2·4	29·7	–	–	–	–	–
ai-$C_{17:1}$	4·7	2·2	6·9	–	–	0·2	–	–	–	–	–
i-2OH-$C_{16:0}$	–	–	6·1	–	–	–	–	–	–	–	–
2OH-$C_{17:0}$	–	–	5·3	–	–	–	–	–	–	–	–
i-$C_{18:0}$	–	–	5·3	–	–	1·4	–	–	–	–	–
i-$C_{18:1}$	–	–	1·3	–	–	0·7	–	–	–	–	–
$C_{18:0}$	–	–	–	2·2	–	2·4	0·7	–	10·1	13·8	–
$C_{18:1}$	3·7	9·7	2·2	3·6	–	8·1	–	–	1·1	–	–
$C_{20:0}$	–	–	–	–	–	–	–	–	2·2	6·3	–
TBSA	–	–	1·5	–	–	–	–	–	–	–	–

* Data from Prauser *et al*. (1997).

† Data from Prauser *et al*. (1997).

‡ Data from Martin *et al*. (1997).

§ Data from Fernández-Garayzábal *et al*. (1995).

‖ Data from Kalakoutskii *et al*. (1967); Kalakoutskii (1989), Schumann *et al*. (1997).

¶ Two isomers of $C_{16:1}$.

DISCUSSION

All three isolates described here, Ben 109^T, Ben 110 and T1-X7^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_4) are a major component of communities of such systems (Hiraishi *et al*., 1998). All three bacteria in this study contain MK-8(H_4), 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 3. Comparative substrate utilization patterns of strains Ben 109^T, Ben 110 and T1-X7^T

Substrate*	T1-X7^T	Ben 109^T	Ben 110
Tween 40	–	+	+
Tween 80	–	+	+
Sucrose	–	+	+
D-Trehalose	–	+	+
3-Methyl glucose	–	+	+
D-Xylose	–	+	+
Pyruvic acid	+	–	–
Adenosine-5′-monophosphate	–	+	+
Lysine	–	+	–
β-Galactosidase	–	+	–

* 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.

Table 4. Levels of DNA–DNA similarity between strains Ben 109^T, Ben 110, T1-X7^T, *Terracoccus luteus*, *Terrabacter tumescens*, *Janibacter limosus* and *Intrasporangium calvum*

Strain	Percentage relatedness to:		
	Ben 109^T	Ben 110	T1-X7^T
Ben 109^T			
Ben 110	89		
T1-X7^T	41	50	
Terracoccus luteus	ND	55	49
Terrabacter tumescens	ND	41	47
Janibacter limosus	40	45	62
Intrasporangium calvum	ND	55	58

ND, Not determined.

Phylogenetically, 16S rDNA sequence analyses reveal that Ben 109^T, Ben 110 and T1-X7^T 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.

All three strains have *m*-A_2pm and type A1γ peptidoglycan, a feature they share with members of the genus *Janibacter*, but none of the other genera mentioned, which all possess LL-A_2PM as their dibasic amino acid and type A3γ 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 109^T, Ben 110 and strain T1-X7^T 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 109^T and Ben 110 to be members of the same genomic species (Stackebrandt & Goebel, 1994; Fox *et al.*, 1992), this value between T1-X7^T and both Ben 109^T 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(H_2) and fatty acid i-$C_{14:0}$ in both Ben 109^T and Ben 110, but not in T1-X7^T, which has MK-7(H_2) and hydroxylated fatty acids, i-$C_{18:0}$ and i-$C_{18:1}$, and other phenotypic differences

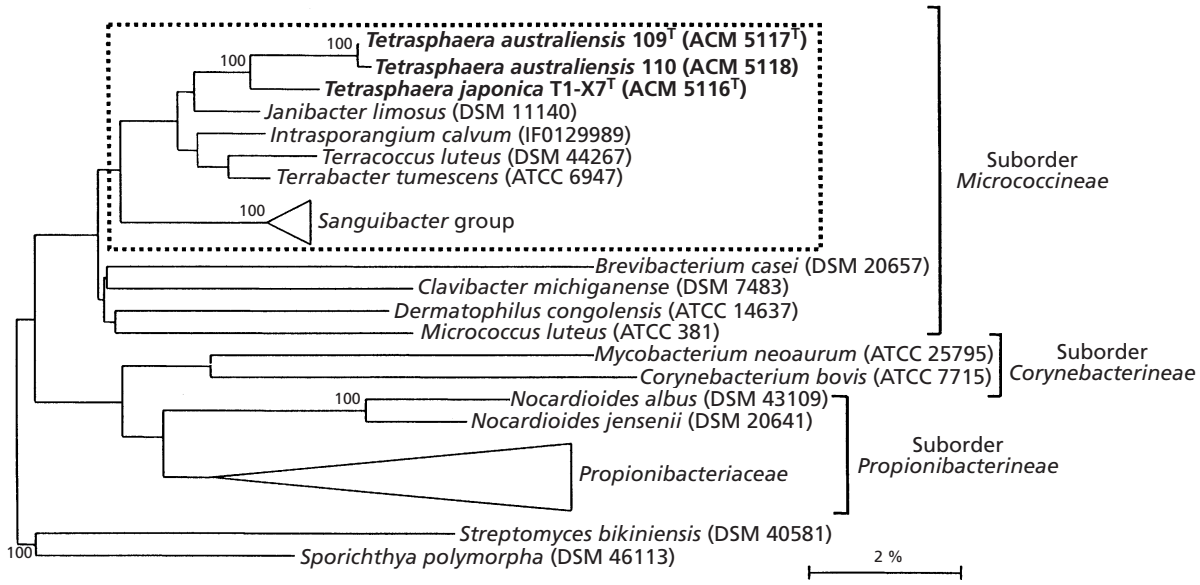

Fig. 2. Dendrogram based on 16S rDNA sequence data indicating the positions of *Tetrasphaera australiensis* (Ben 109^T and Ben 110) *and Tetrasphaera japonica* (T1-X7^T) within the family *Intrasporangiaceae* (shaded box), suborder *Micrococcineae* and their relationship to other members of the order *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 109^T, Ben 110, T1-X7^T and members of the family *Intrasporangiaceae*

Position*	*Intrasporangiaceae*	Ben 109^T	Ben 110	T1-X7^T	*Intrasporangium calvum*	*Sanguibacter*	*Terrabacter*	*Janibacter*	*Terracoccus*
30–553	C-G	C-G	C-G	C-G	C-G	C-G	C-G	C-G	C-G
69–99	G-T	G-T	G-T	G-T	G-T	G-T	G-T	G-T	G-T
140–223	G-C	G-C	G-C	G-C	G-C	G-C	G-C, A-T†, G-T‡	G-T§, A-T‖	G-C
157–164	G-C	T-A	T-A	G-C	G-C	G-C	G-C, T-G†‡	T-G§	G-C
258–268	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T
630	C	C	C	C	C	C	C	C	C
658–748	G-T	A-T	A-T	G-T	G-T	G-T	G-T	G-T	G-T
659–746	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A
660–745	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C
694	G	A	A	G	G	G	G, A†‡	A§‖	G
838–848	C-G	C-G	C-G	C-G	C-G	C-G	C-G	C-G	C-G
839–847	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A
859	C	C	C	C	C	C	C	C	C
1003–1037	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C
1007–1022	C-G	C-G	C-G	C-G	C-G	C-G, C-T¶	C-G, C-T†‡	C-T§‖	C-G
1133–1141	A-T	G-C	G-C	G-C	A-T	A-T	A-T	A-T	A-T
1134–1140	C-G	C-G	C-G	G-C	C-G	C-G	C-G	C-G	A-G#

* *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.

§ *Janibacter limosus* strain DSM 11140 (EMBL Y08539) has G-T instead G-C at position 140–223, T-G instead of G-C at position 157–164, A instead of G at position 694 and C-T instead of C-G at position 1007–1022.

‖ *Janibacter limosus* strain DSM 11141 (EMBL Y08540) has A-T instead of G-C at position 140–223, A instead of G at position 694 and C-T instead of C-G at position 1007–1022.

¶ *Sanguibacter keddieii* strain NCFB 3025^T (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 109^T, Ben 110, T1-X7^T and members of the family *Intrasporangiaceae* within the suborder *Micrococcineae* (Stackebrandt *et al.*, 1997)

Position*	*Micrococcineae*	Ben 109^T	Ben 110	T1-X7^T	*Terrabacter*†	*Intrasporangium calvum*	*Sanguibacter*‡	*Janibacter*§	*Terracoccus luteus*
66–103	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T
70–98	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A
82–87	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C
127–234	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T
449	A	A	A	A	A	A	A	A	A
598–640	T-G	T-G	T-G	T-G	T-G	T-G	T-G	T-G	T-G
600–638	T-G	T-G	T-G	T-G	T-G	T-G	T-G	T-G	T-G
722–733	A-A	A-A	A-A	A-A	A-A	A-A	A-A	A-A	A-A
952–1229	C-G	T-A	T-A	T-A	C-G	C-G	C-G	C-G	C-G
986–1219	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T
987–1218	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T
1059–1198	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A	T-A

* *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 Y085390) 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 *Micrococcineae*, 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* (Pascual *et al*., 1996) also have 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 *Actinobacteria* are obtained and characterized, and as such should be viewed as preliminary. The recent description of *Tessaracoccus* (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-spore-forming. Cultures grow very slowly and utilize a limited number of substrates. Catalase-positive, urease-negative and able to store polyphosphate. Do not produce H_2S or indole. Cannot hydrolyse gelatin, but can utilize propionate, acetate, glucose and arbutin. Cell wall peptidoglycan contains *meso*-diaminopimelic acid (*m*-A_2pm) which characterizes type A1γ murein. The major menaquinone is MK-8(H_4). Mycolic acids are absent. The pH range supporting growth is between 6·0 and 8·0. The main cellular fatty acid is iso-$C_{16:0}$, and ai-$C_{17:0}$, i-$C_{16:1}$, i-$C_{15:0}$ as well as $C_{16:1}$ 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 *Micrococcineae*. 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(H_4) and MK-6(H_4) in addition to the major menaquinone MK8(H_4) and contains an unknown amino phospholipid. It also possesses i-2OH-$C_{16:0}$, 2-OH-$C_{17:0}$, i-$C_{18:0}$, i-$C_{18:1}$ and tuberculostearic acid (TBSA) and lacks both iso-$C_{14:0}$ and $C_{16:0}$. 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.tra.li.en′sis. 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(H_4) as the major menaquinone and MK-8(H_2) is present in trace amounts. In addition, strain Ben 110 possesses a large amount of MK-6(H_4), 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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