Quadrisphaera granulorum gen. nov., sp. nov., a Gram-positive polyphosphate-accumulating coccus in tetrads or aggregates isolated from aerobic granules

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A Gram-positive bacterium, designated strain AG019^T, was isolated by micromanipulation from aerobic granules obtained from a laboratory-scale sequencing batch reactor. This isolate grew axenically as cocci clustered predominantly in tetrads, and was morphologically similar to the dominant organisms observed in the biomass. The morphology also resembled that of the tetrad-forming organisms commonly seen in activated sludge samples. Strain AG019^T was found to be an oxidase-negative, catalase-positive, non-motile aerobe that does not reduce nitrate and grows at temperatures between 15 and 40 °C, with an optimum at 37 °C. The pH range for growth was 5.0–9.0, with an optimum at pH 7.5. Strain AG019^T contained a peptidoglycan with directly cross-linked *meso*-diaminopimelic acid (type A1γ) and lacked mycolic acids. The G + C content of the DNA was 75 mol%. Menaquinone MK-8(H₂) was the major isoprenoid quinone. The bacterium stained positively for intracellular polyphosphate granules but not for poly-β-hydroxyalkanoates. It produced capsular material and showed autoaggregation ability. Phenotypic and 16S rRNA gene analyses showed that the bacterium differed sufficiently from its closest phylogenetic relatives, namely members of the suborder *Frankineae*, which includes the genera *Geodermatophilus*, *Blastococcus*, *Frankia*, *Sporichthya*, *Acidothermus* and *Microsphaera*, that it is proposed that it be placed in a novel genus, *Quadrisphaera*, as *Quadrisphaera granulorum* gen. nov., sp. nov. The type strain is AG019^T (= ATCC BAA-1104^T = DSM 44889^T).

The occurrence of coccoid bacteria with a distinctive tetrad morphology first surfaced in activated sludge systems in the early 1990s (Cech & Hartman, 1990). These were Gram-negative cocci and were commonly seen in reactors fed with an acetate/glucose mixture. Since then, tetrad-forming organisms (TFOs) with a similar morphological description have been reported in other activated sludge systems. These organisms belong to several novel genera that span both the Gram-positive and Gram-negative lineages (Maszenan, 2000; Seviour *et al.*, 2000; Tsai & Liu, 2002).

TFOs have also been detected during routine microscopic examination of aerobically grown microbial granules cultivated in a laboratory-scale sequencing batch reactor (Dulekgurgen *et al.*, 2003). Aerobic granules are a recent innovation in biological wastewater treatment and are self-immobilized aggregates of bacteria with a strong and compact structure, good settling ability, high biomass retention and an ability to handle high organic loading rates (Moy *et al.*, 2002). The granules are also metabolically versatile, have been cultivated on different biodegradable organic substrates (Jiang *et al.*, 2002, 2004; Moy *et al.*, 2002; Pan *et al.*, 2004; Tay *et al.*, 2001) and are capable of nitrification, denitrification and polyphosphate accumulation (Dulekgurgen *et al.*, 2003; Jang *et al.*, 2003; Lin *et al.*, 2003; Tay *et al.*, 2002; Zeng *et al.*, 2003).

This paper describes the characterization of a Gram-positive TFO, designated strain AG019^T, isolated from aerobic granules. The results of phenotypic and phylogenetic

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Abbreviation: TFO, tetrad-forming organism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AG019^T is AY831385.

Tables comparing the signature nucleotides of the 16S rRNA gene for the suborder *Frankineae* and strain AG019^T are available as supplementary data in IJSEM Online.

analyses support the classification of strain AG019^T as a novel genus within the family *Frankiaceae*.

The aerobic granules were cultivated in a laboratory-scale sequencing batch reactor as described previously, with synthetic wastewater containing acetate as the sole carbon source (Moy *et al.*, 2002). Granule samples were harvested 4 weeks after reactor startup, and were disrupted at 2500 r.p.m. for 3 min with a Mini Beadbeater (Biospec Products). Microscopic observations (BX60 apparatus; Olympus) of these granules revealed that the microbial community was dominated by coccoid cells that clustered mostly in tetrads. These coccoid cells were retrieved from the disintegrated biomass using a Skerman micromanipulator (Skerman, 1968) and transferred to GS agar (Williams & Unz, 1985) plates for incubation at 25 °C (Williams & Unz, 1985). Colonies arising from micromanipulated cells were transferred several times to GS agar plates to obtain pure cultures; culture purity was confirmed microscopically (BX60; Olympus) by examining cells from single colonies. An axenic culture of strain AG019^T was preserved at -80 °C in GS medium (Williams & Unz, 1985) containing 20 % glycerol.

Strain AG019^T possessed the distinctive morphology of TFOs, i.e. cocci arranged in tetrads or clusters (Fig. 1). Strain AG019^T was slow-growing and took 7 days to appear as visible colonies on a GS agar plate; it was probably an aerobe, as no growth occurred down the line of inoculation in stab culture. The strain grew at temperatures between 15 and 40 °C, with an optimum at 37 °C, and grew at pH values between 5.0 and 9.0, with an optimum at pH 7.5. The cells stained Gram-positive with the modified Gram-stain method of Hucker (Smibert & Krieg, 1994); this was also confirmed by the absence of stringiness with 3 % KOH treatment (Buck, 1982). No flagella were detected, and the motility test indicated that strain AG019^T was non-motile.

Fig. 1. Scanning electron micrograph of strain AG019^T showing cocci in tetrad and cluster arrangement. Bar, 3 μm.

Polyphosphate granules were observed when cells were grown aerobically with glucose, acetate or propionate as the sole carbon source, but polyhydroxyalkanoate granules were not detected when cells were grown anaerobically using the dual staining method of Rees *et al.* (1992). No endospores were detected. Capsular materials were observed with the Indian ink stain (Difco).

A flocculation assay was performed on strain AG019^T, as described previously (Malik *et al.*, 2003). Strain AG019^T was inoculated into 200 ml GS medium, then incubated at 20 °C with shaking at 100 r.p.m. The optical density (OD) of the mixed suspension was measured at a wavelength of 600 nm as initial OD (OD_{initial}). The cell suspension was allowed to stand at 20 °C, then the supernatant layer was carefully pipetted at different standing times for OD measurement. The aggregation index was calculated on the basis of the OD value (OD_t) at a predefined standing time (*t*), as follows:

$$\text{Aggregation index (\%)} = \frac{\text{OD}_{\text{initial}} - \text{OD}_t}{\text{OD}_{\text{initial}}} \times 100$$

Flocculation-assay results showed that strain AG019^T had high autoaggregation ability. The aggregation index reached 25 % after a standing time of between 150 and 210 min.

The physiological and biochemical characteristics of strain AG019^T are presented in the descriptions of the genus and species. Enzyme profiles and biochemical characteristics of strain AG019^T were determined using the API ZYM and API 20E systems according to the manufacturer's instructions (bioMérieux). Carbon-substrate utilization tests were performed with the Biolog GN and GP systems (Special Diagnostics). The cells were catalase-positive but oxidase- and urease-negative, as determined by the method of Smibert & Krieg (1994). The G + C content of the genomic DNA was 75 mol%, as determined by using the reverse HPLC method (Schumann *et al.*, 1997).

The peptidoglycan, menaquinone, cellular fatty acid and polar lipid compositions were analysed as described by Schumann *et al.* (1997). Strain AG019^T possessed type A1γ peptidoglycan with *meso*-diaminopimelic acid as the diagnostic diamino acid (Schleifer & Kandler, 1972). The cells lacked mycolic acid, but contained four isoprenoid quinones, MK-8(H₂), MK-7(H₂), MK-6(H₂) and MK-9(H₂), in the ratio 88:3:2:1. The polar lipids present included diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol; the cellular fatty acid profiles of strain AG019^T were dominated by 12-methyl tetradecanoic acid and hexadecanoic acid (Table 1).

The almost-complete 16S rRNA gene of strain AG019^T was amplified and sequenced using methods described previously (Maszenan *et al.*, 1997). A sequence of 1403 nucleotide bases was obtained in both directions, corresponding to positions 20–1471 of *Escherichia coli* according to the nomenclature of Winker & Woese (1991), and was manually aligned against reference sequences of close relatives by

Table 1. Cellular fatty acid composition of strain AG019^T

Cellular fatty acid composition	Content (%)
Tetradecanoic acid (C _{14:0})	3.2
13-Methyl tetradecanoic acid (i-C _{15:0})	4.0
12-Methyl tetradecanoic acid (ai-C _{15:0})	56.7
Pentadecanoic acid (C _{15:0})	3.6
14-Methyl pentadecanoic acid (i-C _{16:0})	4.1
Hexadecanoic acid (C _{16:0})	14.9
15-Methyl hexadecanoic acid (i-C _{17:0})	2.2
14-Methyl hexadecanoic acid (ai-C _{17:0})	4.1
cis-Octadecanoic acid (C _{18:1})	5.6

using the alignment editor in BioEdit (Hall, 1997). Sequence analysis was performed using BLAST (Altschul *et al.*, 1997), CLUSTAL W (Thompson *et al.*, 1994), SIMILARITY_RANK and SUGGEST_TREE in the Ribosomal Database Project, version 8.0 (Maidak *et al.*, 1997). Distance analysis was performed on a final dataset that contained an unambiguous alignment of 1314 bases of strain AG019^T and its closest relatives. A phylogenetic tree was constructed from evolutionary distances by using the FITCH program in PHYLIP (Felsenstein, 1985). Bootstrap confidence values were obtained with 100 resamplings.

To our knowledge, this is the first study to use micro-manipulation on aerobic granules for isolating TFOs. Conventional isolation techniques such as serial dilution and the spread-plate method have a low success rate for the isolation of TFOs because these micro-organisms grow slowly, and fast-growing bacterial cells often outgrow them (Maszenan, 2000; Seviour *et al.*, 2000). Previous studies of

TFOs in activated sludge systems have resulted in the isolation of several novel genera of the *Actinobacteria*, some of which are thought to play a major role in biological phosphorus removal in activated sludge plants (Christensson *et al.*, 1998; Seviour *et al.*, 2000). Strain AG019^T stained positively for polyphosphate when grown under aerobic conditions using either glucose or acetate as the sole carbon source, but no polyhydroxyalkanoate accumulation was observed under anaerobic conditions with acetate. Flocculation studies also show that strain AG019^T can autoaggregate with an aggregation index of 25 % after 120 min. The ability both to autoaggregate and synthesize capsular material might confer on strain AG019^T a selective advantage over other bacteria in aerobic granular sludge systems in allowing them to overcome cell washout and thus remain in the reactor, and also to avoid predation from bacteria scavengers such as ciliates and protozoa (Cech *et al.*, 1994; Seviour *et al.*, 2000).

Analysis of the 16S rRNA gene from strain AG019^T revealed that it is a member of the Gram-positive bacteria in the high-G+C-content group *Actinobacteria* classis nov. in the domain *Bacteria* (Stackebrandt *et al.*, 1997). Pairwise comparison of 16S rRNA gene sequences revealed that strain AG019^T was 95 % similar to *Kineococcus*-like bacteria and *Sporichthya*, 93 % similar to the type strain of *Kineococcus aurantiacus*, less than 94 % similar to members of the genera *Frankia* and *Blastococcus*, and 93 % similar to members of the genera *Acidothermus*, *Geodermatophilus* and *Microsphaera*, as shown in Fig. 2. Strain AG019^T is related to members of the genus *Microsphaera* (Yoshimi *et al.*, 1996), which belongs to the suborder *Frankineae*. However, strain AG019^T is different from the other TFOs in the

Fig. 2. Phylogenetic tree based on analysis of 16S rRNA gene sequences of strain AG019^T and representatives of the *Actinobacteria*. All sequences used in the analysis were obtained from GenBank. Bootstrap values, expressed as a percentage of 100 replications, are shown at the branching points. Bar, 10 substitutions per 100 nt.

Actinobacteria group, which includes the genera *Tessaracoccus* (Maszenan *et al.*, 1999a), *Friedmanniella* (Maszenan *et al.*, 1999b) and *Tetrasphaera* (Maszenan *et al.*, 2000).

Using the taxonomic scheme of Stackebrandt *et al.* (1997), it is clear that strain AG019^T fits readily within the suborder *Frankineae*, except that A–T is found instead of G–C at nucleotide positions 127–234, and that A–T is present instead of G–C at positions 141–222 (see Table S1 available as supplementary data in IJSEM Online). The most closely related genera show complete concurrence with the scheme of Stackebrandt *et al.* (1997) for the suborder *Frankineae*. On the other hand, the 16S rRNA structure of strain AG019^T also contains several nucleotide pairs that are different from those of members of the families *Frankiaceae*, *Geodermatophilaceae*, *Microsphaeraceae*, *Sporichthyaceae* and *Acidothermaceae* (see Table S2 available as supplementary data in IJSEM Online), lending support to the notion that strain AG019^T belongs to a novel genus in the suborder *Frankineae*.

Strain AG019^T differs from the cells of the genus *Frankia*, which occur as chains of cocci, while *Geodermatophilus* and *Blastococcus* are pleomorphic and occur as rods and cocci. *Sporichthya* and *Microsphaera*, in the suborder *Frankineae*, are cocci. However, the cell-wall peptidoglycan of *Sporichthya* contains LL-diaminopimelic acid and the menaquinones MK-9(H₆) and MK-9(H₈) (Rainey *et al.*, 1993), while strain AG019^T possesses meso-diaminopimelic acid and MK-8(H₂) as its dominant menaquinones. Although strain AG019^T, members of the genus *Kineococcus* (Yokota *et al.*, 1993; Phillips *et al.*, 2002) and members of the genus *Microsphaera* (Yoshimi *et al.*, 1996) contain meso-diaminopimelic acid in their cell-wall peptidoglycan, strain AG019^T has a major menaquinone profile that is different from that of *Microsphaera* and *Kineococcus*. Members of the genera *Microsphaera* and *Kineococcus* possess MK-8(H₄) and MK-9(H₂), respectively, whereas MK-8(H₂) is the predominant quinone in strain AG019^T.

Pairwise comparison of 16S rRNA gene sequences revealed that strain AG019^T was 95 % similar to *Kineococcus*-like bacteria and *Sporichthya*, 93 % similar to the type strain of *Kineococcus aurantiacus*, less than 94 % similar to members of the genera *Frankia* and *Blastococcus*, and 93 % similar to members of the genera *Acidothermus*, *Geodermatophilus* and *Microsphaera* (Fig. 2). These results, taken together with the phylogenetic analysis described earlier, show that strain AG019^T is different from members of the genera *Frankia*, *Blastococcus*, *Microsphaera*, *Acidothermus*, *Sporichthya* and *Geodermatophilus* (Table 2).

Strain AG019^T is only 97.2 % similar to its closest phylogenetic relative, strain G10, which originated from Namibia and which had been previously reported to be equidistantly related to *Geodermatophilus obscurus*, *Frankia* and *Acidothermus cellulolyticus* (Eppard *et al.*, 1996). In this study, strains AG019^T and G10 form a coherent group.

However, on the basis of chemotaxonomic properties such as the presence of a greenish to dark pigment, faint glossy colonies and a reproduction mechanism involving binary fission and budding, as described by Eppard *et al.* (1996), strain G10 appears to be more closely related to the genus *Geodermatophilus* than to strain AG019^T. Thus the phylogenetic evidence and chemotaxonomic properties presented so far support the placement of strain AG019^T in a novel genus, for which we propose the name *Quadrisphaera*, with *Quadrisphaera granulorum* as the type species.

Description of *Quadrisphaera* gen. nov.

Quadrisphaera (Qua.dri.sphae'ra. L. pref. numer. adj. *quadr-* four; L. fem. n. *sphaera* a ball, globe, sphere; N.L. fem. n. *Quadrisphaera* fourfold balls, coccus in tetrad).

Gram-positive, non-spore-forming cocci, 1.2–3.0 µm in diameter, occurring in tetrad arrangement, fitting the morphological description of TFOs. MK-8(H₂) is the predominant menaquinone. The phylogenetic position is in the family *Frankiaceae*.

The type species is *Quadrisphaera granulorum*.

Description of *Quadrisphaera granulorum* sp. nov.

Quadrisphaera granulorum (gra.nu.lo'rum. L. gen. pl. neut. n. *granulorum* from, or of, granules).

Results obtained with the Biolog GN and GP systems and the API 20E system showed that strain AG019^T has the following characteristics (in addition to those described in the genus description). Utilizes α-cyclodextrin, α-DL-glycerol phosphate, Tween 40, arbutin, glucose 1-phosphate, Tween 80, glucose 6-phosphate, adonitol, L-arabinose, D-arabitol, glucuronamide, cellobiose, D-psicose, D-mannitol, D-melezitose, D-melibiose, L-serine, methyl β-D-glucoside, psicose, D-xylose, methyl pyruvate, pyruvate, 2-aminoethanol, mono-methyl succinate, glycerol, L-serine, turanose and glycerol. Strain AG019^T cannot metabolize the following: β-cyclodextrin, dextrin, glycogen, inulin, mannan, N-acetyl D-galactosamine, N-acetylglucosamine, N-acetylmannosamine, amygdalin, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-glucuronic acid, α-D-glucose, m-inositol, α-D-lactulose, α-lactose, maltose, D-mannitol, D-mannose, D-melezitose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-raffinose, L-rhamnose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-trehalose, xylitol, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, itaconic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, lactamide, D-lactic acid methyl ester, D-malic acid, L-malic acid, propionic acid, succinamic acid, succinic

Table 2. Comparative phenotypic characteristics of strain AG019^T and members of the suborder *Frankineae*

All isolates are Gram-positive. Symbols: +, positive test result; –, negative test result; +/–, variable test result; ND, not determined.

Phenotypic characteristic	<i>Frankia</i> *	<i>Geodermatophilus</i> *	<i>Blastococcus</i> *	<i>Microsphaera</i> †	<i>Sporichthya</i> ‡	<i>Acidothermus</i> §	Strain AG019 ^T
O ₂ requirement	Aerobic to microaerophilic	Aerobic	Aerobic to microaerophilic	Aerobic	Aerobic	Aerobic	Aerobic
Cell morphology	Actinomycetes producing septate and filamentous mycelium	Motile rods, irregularly shaped aggregates of coccoid cells	Motile rods, irregularly shaped aggregates of coccoid cells	Cocci	Aggregate of coccoid cells in tetrad arrangements	Slender rods and floccules	Aggregates of coccoid cells in tetrad arrangements
Motility	–, non-motile	+, motile	–, non-motile	–, non-motile	+/–	–, non-motile	–, non-motile
Habitat	Soil, plant roots	Desert soil, sea	Sea	Sugar waste activated sludge	Soil	Hot spring	Aerobic granular sludge
Optimum growth temperature (°C)	20–36	26	ND	25	20–30	50–60	37
Optimum growth pH	ND	ND	ND	7.0	ND	5.0	7.5
Growth temperature range (°C)	10–37	10–37	ND	10–35	15–37	37–70	15–40
Growth pH range	6.4–6.8	ND	ND	5.0–9.0	ND	3.5–7.0	5.0–9.0
Oxidase	ND	ND	ND	–	ND	–	–
Catalase	+	+	ND	+	ND	+	+
Cell-wall type	<i>meso</i> -Diaminopimelic acid (type III)¶; lacks mycolic acid	<i>meso</i> -Diaminopimelic acid (type III); lacks mycolic acid	ND	<i>meso</i> -Diaminopimelic acid	LL-Diaminopimelic acid	<i>meso</i> -Diaminopimelic acid	<i>meso</i> -Diaminopimelic acid; lacks mycolic acid
Major menaquinone	MK-9(H ₄)	MK-9(H ₄)	ND	MK-8(H ₄)	MK-9(H ₆), MK-9(H ₈)	ND	MK-8(H ₂)
Polar lipid(s)#	PIM, PI, DPG	PII	ND	ND	PI, PG, DPG	ND	DPG, PG, PI
Nitrate reduction	ND	+/–	ND	ND	+	–	–
DNA G + C content (mol%)	66–71	73–75	70–75	68	70–71	61	75

*Normand *et al.* (1996).

†Yoshimi *et al.* (1996).

‡Rainey *et al.* (1993), Tamura *et al.* (1999).

§Mohagheghi *et al.* (1986).

||Note: *Sporichthya brevicatena* spores exhibit motility when suspended in sterile distilled water.

¶Type III cell walls contain *meso*-diaminopimelic acid, which contains glutamic acid, alanine, glucosamine and muramic acid.

#Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PII, phosphatidylethanolamine and/or phosphatidylmethanolamine; PIM, phosphatidylinositol mannosides.

acid, *N*-acetylglutamic acid, bromosuccinic acid, alaninamide, *D*-alanine, *L*-alanine, *L*-alanyl-glycine, *L*-asparagine, glycyl-*L*-glutamic acid, *L*-pyroglutamic acid, putrescine, 2,3-butanediol, glycyl-*L*-aspartic acid, *L*-histidine, hydroxyl-*L*-proline, *L*-leucine, *L*-ornithine, *L*-phenylalanine, *L*-proline, *L*-pyroglutamic acid, *D*-serine, *L*-threonine, *DL*-carnitine, γ -aminobutyric acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, phenylethylamine or putrescine. The following acid and acid derivatives are utilized by strain AG019^T: glucuronic acid, α -ketobutyric acid, α -ketovaleric acid, *DL*-lactic acid, *L*-aspartic acid, *L*-glutamic acid, uroconic acid and pyruvic acid. The enzyme activities detected by both API ZYM and API 20E are as follows: esterase, esterase lipase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase. Activities of the following enzymes are not detected by API ZYM: alkaline phosphatase, lipase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Activities of the following enzymes are not detected by API 20E: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase. Strain AG019^T does not produce H₂S or indole. It is Voges-Proskauer-negative, does not produce acetoin and does not reduce nitrate to nitrite. It is catalase-positive but oxidase-negative. The genomic G + C content is 75 mol%.

The type strain, AG019^T (=ATCC BAA-1104^T=DSM 44889^T), was isolated from aerobic granules.

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