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NOTE

Quadricoccus australiensis gen. nov., sp. nov., a β-proteobacterium from activated sludge biomass

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A Gram-negative coccus, designated strain Ben 117^T, was obtained in axenic culture by micromanipulation from an Australian activated sludge biomass sample, which had been subjected to chlorination in order to alleviate problems associated with foaming and bulking. This isolate was a strict aerobe and grew in axenic culture, also appearing in biomass samples as cocci or clusters of cocci in tetrads, thus resembling the morphotype 'G-bacteria' seen commonly in activated sludge samples. Strain Ben 117^T was non-motile, aerobic, oxidase-negative and catalase-positive and grew between 15 and 30 °C, with an optimum of 25–30 °C. The pH range for growth was between 6·0 and 8·5, with an optimum of 7·5–8·5. The isolate stained positively for intracellular polyphosphate and poly-β-hydroxybutyrate and its G+C content was 67 mol%. 16S rDNA sequence analysis suggests that strain Ben 117^T is phylogenetically different from members of the genera *Amaricoccus*, Gram-negative 'G-bacteria' isolated previously in this laboratory. Ben 117^T is a member of the *Rhodocyclus* group in the *β-Proteobacteria* and equidistantly placed (similarity value of 95%) between *Ferribacterium limneticum* and *Dechloromonas agitata* (mean similarity value of 92% with the genus *Rhodocyclus*). Based on phenotypic and phylogenetic evidence, it is proposed that strain Ben 117^T be designated a novel species in a new genus, *Quadricoccus australiensis* gen. nov., sp. nov.; the type strain is Ben 117^T (= NCIMB 13738^T = CIP 107055^T).

Keywords: 'G-bacteria', activated sludge, *Proteobacteria*, *Quadricoccus* gen. nov., Gram-negative coccus

All enhanced biological phosphorus removal (EBPR) processes share an operational feature in which the biomass is cycled through alternating aerobic/anaerobic regimes. These conditions are considered necessary to provide polyphosphate-accumulating bacteria (PAB) with a selective advantage based on their ability to assimilate substrates anaerobically into intracellular storage compounds that can then be used as energy sources in the aerobic reactor (Mino *et al.*, 1998). However, the microbiology of EBPR processes is still poorly understood despite considerable efforts directed at attempting to identify PAB (Bond & Rees, 1999). Consequently, when these systems fail, as they often do, identifying the reasons and taking appropriate remedial action is currently not possible. Cech & Hartman (1990) described one possible cause for EBPR failure in a small reactor. With a glucose feed, they noticed that Gram-negative cocci in tetrads dominated the biomass and postulated that these bacteria, which they called 'G-bacteria', were outcompeting the PAB. These were cultured but incorrectly identified, a mistake which was subsequently rectified when Maszenan *et al.* (1997) showed that these cocci belonged to a novel genus, *Amaricoccus*.

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Abbreviations: EBPR, enhanced biological phosphorus removal; PAB, polyphosphate-accumulating bacteria; PHA, polyhydroxyalkanoate; polyP, polyphosphate.

The GenBank accession number for the 16S rRNA gene sequence of *Quadricoccus australiensis* strain Ben 117^T is AY007722.

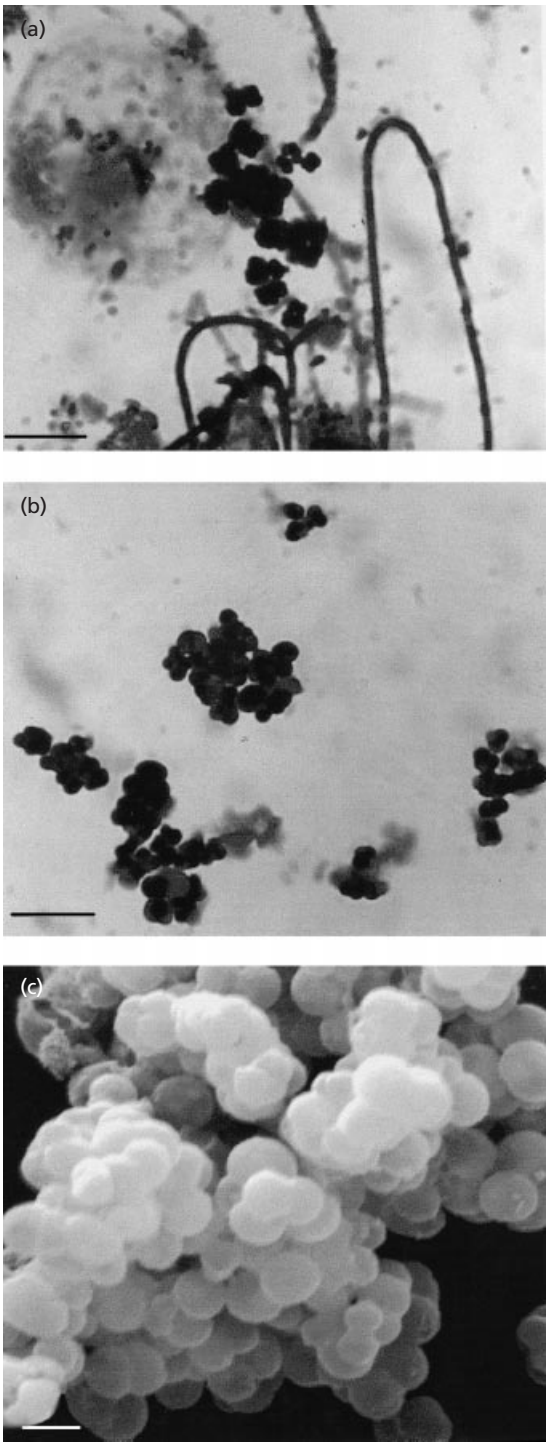

Fig. 1. (a) Cocci in tetrads seen in biomass samples from Subiaco, the source of strain Ben 117^T (bar, 10 μm). (b) Axenically grown cells of strain Ben 117^T after Gram staining (bar, 10 μm). (c) SEM of axenic culture of strain Ben 117^T showing clustered cocci (bar, 1 μm).

Since then, other cocci, microscopically indistinguishable from *Amaricoccus*, have been described in activated sludge and shown to belong to several different genera, some of which are previously undescribed, in the high-G+C-containing, Gram-positive bacteria (Maszenan *et al*., 1999a, b, 2000) and the α- and β-*Proteobacteria* (A. M. Maszenan, unpublished results; Nielsen *et al*., 1999).

In this paper, strain Ben 117^T, a coccus which fits the morphological description of 'G-bacteria', isolated from a sample of activated sludge biomass from a treatment plant in Subiaco (Western Australia) using micromanipulation (Skerman, 1968), is described. The sample was collected from an aeration tank of the plant that had been subjected to chlorination in an attempt to control the overgrowth of filamentous bacteria associated with bulking and foaming. Several media routinely used for culturing bacteria from activated sludge were tested, but only GS agar (Maszenan *et al*., 1997) was successful in isolating strain Ben 117^T. After 3–4 weeks incubation on GS agar, the micromanipulated cocci were checked microscopically for contamination from any unwanted faster-growing bacteria and were removed from contaminants by micromanipulation. Visible colonies that developed were streaked onto fresh GS plates and culture purity was confirmed microscopically. Axenic cultures of strain Ben 117^T were preserved at −80 °C in GS medium containing 20 % glycerol. Isolated cells of strain Ben 117^T were coccoid shaped (2·2–4·5 μm in diameter) and fitted the morphological description of 'G-bacteria', i.e. cocci in single or clustered tetrads (Fig. 1). Strain Ben 117^T took 7 d to produce visible white fluffy colonies when grown on GS agar at 25 °C. Axenically and *in situ*, strain Ben 117^T stained Gram-positively with modified Hucker stain (Fig. 1), but produced cell stringiness with 3 % KOH treatment, which implied that it had a Gram-negative type wall. Endospores were never detected. Cells were non-motile and flagella were not observed. Intracellular deposits of both polyphosphate (polyP) and polyhydroxyalkanoate (PHA) could be demonstrated when cells were cultured on GS medium containing glucose, acetate and propionate as sole carbon source under aerobic conditions, using the dual staining method of Rees *et al*. (1992). Strain Ben 117^T grew optimally at 25–30 °C on GS medium, but did not grow at 10 °C or above 37 °C. Optimum pH for growth was 7·5–8·5; no growth occurred at pH 5·5 or 9·0.

Strain Ben 117^T utilized substrates as detailed in the taxonomic description, determined with the BIOLOG GN and GP systems incubated at 25 °C for 1 week. Enzymes detected in strain Ben 117^T using the API ZYM and Microbact 24E systems were also included in the taxonomic description after incubation at 25 °C for 4 and 48 h, respectively. Cells were oxidase-negative, but urease- and catalase-positive. No H_2S or acetoin were produced (i.e. Voges–Proskauer-negative). The DNA G+C composition determined using the method of Owen & Lapage (1976) for strain Ben 117^T was 67 mol %.

An almost complete 16S rRNA sequence for strain Ben 117^T (1490 bases) was obtained using sequencing

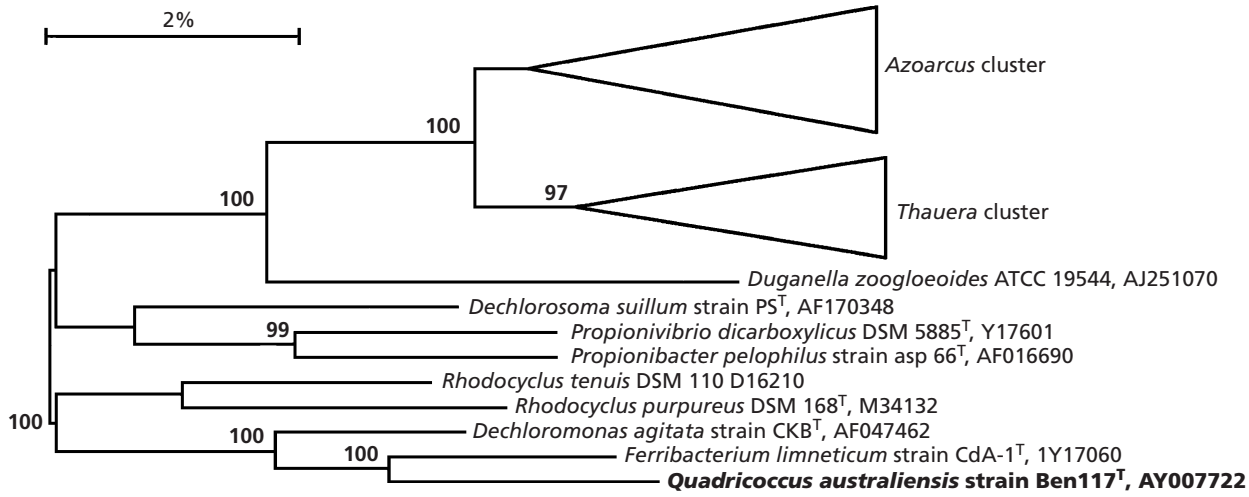

Fig. 2. Phylogenetic tree based on 16S rDNA sequence data of Ben 117 ^{T} showing its relationship to other members of the *β-Proteobacteria* using the neighbour-joining method. All sequences were obtained from the Ribosomal Database Project. Bootstrap values are given in bold. Bar, 2 nt substitutions per 100 nt. *Rhodobacter sphaeroides* ATCC 17023^T (GenBank accession number X53854) and *Gluconacetobacter hansenii* NCIB 8746^T (GenBank accession number X75620), members of the *α-Proteobacteria*, were used as the outgroup (data not shown).

and sequence analysis protocols described previously (Maszenan *et al*., 1997). Initially, analysis indicated that Ben 117^T was a member of the phylum *Proteobacteria* (Woese *et al*., 1984; Woese, 1987). Further detailed analysis with several representative sequences from members of the *Proteobacteria* showed that Ben 117^T, *Ferribacterium limneticum* (Cummings *et al*., 1999) and *Dechloromonas agitata* (Bruce *et al*., 1999; Coates *et al*., 1999) grouped together forming a deep branch within the *Rhodocyclus* group of *β-Proteobacteria*. Transversion analysis did not change the relative position of strain Ben 117^T and bootstrap analysis demonstrated that these phylogenetic relationships are stable with a high confidence value. A dendrogram showing this relationship is presented in Fig. 2.

Phenotypic and phylogenetic results on strain Ben 117^T presented in this report once again highlight the existence of an enormous taxonomic diversity of cocci fitting the morphological description of 'G-bacteria' in activated sludge biomass. Strain Ben 117^T is phylogenetically distinct from those morphotypes already described (Maszenan *et al*., 1999a, b, 2000; Nielsen *et al*., 1999) and is the only cultured representative of the 'G-bacteria' reported so far to be a member of the *β-Proteobacteria*. Strain Ben 117^T is almost equidistantly placed (similarity value of 95%) between *F. limneticum* and *D. agitata*. The closest validated taxa are members of the genus *Rhodocyclus* (mean similarity value of 92%). Such a close relationship would normally be used to assign strain Ben 117^T as a member of the genus *Ferribacterium* or *Dechloromonas*. However, this cannot be supported because of the considerable differences in phenotypic characteristics (Table 1). The present taxonomic status of the *Rhodocyclus* group is considered unsatisfactory and incoherent and in need of review and revision (Willems *et al*., 1991; Trüper & Imhoff, 1992; Hurek *et al*., 1997). It is apparent that members of the *Rhodocyclus* group are not separated from each other by great phylogenetic depth. For example, the similarity of 16S rDNA sequences between members of *Azoarcus* and *Thauera* is 94% and that between *Dechloromonas* and *Rhodocyclus is* 93%. Morphologically, strain Ben 117^T is non-motile and cells are coccoid, whereas *Dechloromonas* and *Ferribacterium* are motile and rod-shaped. Moreover, strain Ben 117^T is aerobic, whereas both *Dechloromonas* and *Ferribacterium* are anaerobic. It will therefore be necessary to apply a polyphasic approach to the taxonomy of members of the *Rhodocyclus* group and both genera, although our findings support the view that strain Ben 117^T represents a novel taxon. It is proposed that strain Ben 117^T be designated a novel species in a newly created genus, *Quadricoccus australiensis* gen. nov., sp. nov., within the *Rhodocyclus* group of the *β-Proteobacteria*.

The significance of strain Ben 117^T and the other 'G-bacteria' in activated sludge is not yet clearly understood, although many strains appear to be well-suited to such an ecosystem (Seviour *et al*., 2000) in their shared abilities to synthesize intracellular storage compounds as a means of surviving the inevitable periods of nutrient limitation. Thus, Ben 117^T can synthesize both polyP and PHA aerobically, whereas both *Amaricoccus* (Maszenan *et al*., 1997) and '*Defluvicoccus*' (A. M. Maszenan, unpublished), members of the *α-Proteobacteria*, accumulate PHA and some of the Gram-positive 'G-bacteria' store polyP (Maszenan *et al*., 1999a, b, 2000). Some members of the *β-Proteobacteria* in the *Rhodocyclus* group have been suggested to play an important role in EBPR (Snaidr *et al*., 1997; Bond *et al*., 1999a, b, c; Hesselmann *et al*., 1999) based on molecular data from

Table 1. Comparative characteristics of strain Ben 117^T and members of the genera *Thauera*, *Azoarcus*, *Ferribacterium*, *Dechloromonas*, *Nitrosospira*, *Rhodocyclus* and strain Ben 117^{T}of the *β-Proteobacteria* group

Characteristic	*Thauera**	*Azoarcus*†	*Ferribacterium*‡	*Dechlorimonas*§	*Nitrosospira*‖	*Rhodocyclus*#	Strain Ben 117^T
Morphology (cell diameter)	Rod-shaped (0·5–1·5 μm)	Straight to slightly curved rod, singly and in pairs (0·4–1·0 μm)	Rod-shaped, straight or slightly curved cells (1·4–2·0 μm)	Short rod (0·5–2·0 μm)	Spiral, curved and lobate and cocci observed in stationary phase (0·3–0·4 μm)	Half circle to circle and curved rod (0·3–0·7 μm)	Coccoid cells (2·2–4·5 μm)
PHB	+	+	+	ND	ND	ND	+
Slime formation	+	ND	ND	ND	+	+/−, − for *R. purpureus*	+
Gelatin liquefaction	+/−	ND	ND	ND	+	−	+
G+C content (mol%)	64–68	62–68	ND	ND	52–56	65–72	67
Motility	+	+, one polar flagellum	+	Motile+polar flagellum	+, by peritrichous flagella or non-motile	−/+	−
Natural habitat	Activated sludge, sewage, anaerobic sludge	Saline-sodic soils, refinery oil sludge	Sediment/water interface	Soil sample	Terrestrial and freshwater environment	*R. purpureus* from swine waste lagoon	Activated sludge
Oxidase	+	+	ND	ND	ND	ND	−
Catalase	+	+ (except for *A. anaerobius*)	ND	ND	ND	ND	+
O_2 requirement	Facultative anaerobe	Aerobe, microaerophilic growth observed	Obligate anaerobe	Facultative anaerobe	Aerobe	Aerobe, microaerobic in dark	Aerobe
Optimum growth temperature (°C)	25–30	28	ND	35	25–30	30	25–30
Optimum growth pH (range)	7·0 (7·0–7·4)	7·0–7·2 (6·5–8·2)	ND	7·5 with 1% NaCl	7·5 (7·0–8·0)	7·2 (6·5–7·5)	7·5–8·5
Urease	ND	− (except for *A. indigens*)	ND	ND	ND	ND	+
Citrate utilization	+	−	ND	ND	ND	−	−
Nitrate reduction	ND	−	ND	ND	+	ND	−
Indole	ND	−	ND	ND	ND	ND	−
Voges–Proskauer	ND	− (except for *A. indigens*)	ND	ND	ND	ND	+

ND, Not determined.

* Data obtained from Macy *et al.* (1993), Anders *et al.* (1995), Foss & Harder (1998) and Scholten *et al.* (1999).

† Data obtained from Reinhold-Hurek *et al.* (1993), Zhou *et al.* (1995) and Song *et al.* (1999).

‡ Data obtained from Cummings *et al.* (1999).

§ Data obtained from Bruce *et al.* (1999) and Coates *et al.* (1999).

‖ Data obtained from Meijer *et al.* (1999) and Pfennig (1978).

Data obtained from Springer *et al.* (1998).

mixed culture systems. Thus, the capacities of strain Ben 117^T to store both polyP and PHA when grown aerobically in pure culture are properties that are consistent with it having some role in EBPR systems. However, whether PHA can be synthesized anaerobically and then used as a carbon or energy source under aerobic conditions as the models demand (Mino *et al*., 1998) remains to be seen.

Description of *Quadricoccus* gen. nov.

Quadricoccus (Quad'ri.coc.cus, M.L. *quadro* four; L. masc. n. *coccus* sphere; N.L. n. *Quadricoccus* four spherical cells).

Large Gram-negative, non-spore-forming cocci, 2·2–4·5 μm in diameter, occurring in tetrads, fitting the morphological description of 'G-bacteria'. Cells are aerobic, non-motile and no flagella are observed. Both polyP and PHA are synthesized under aerobic conditions of growth. *Quadricoccus* is oxidase-negative, but catalase-positive. DNA G+C composition is 67 mol%. Type species is *Quadricoccus australiensis*.

Description of *Quadricoccus australiensis* sp. nov.

Quadricoccus australiensis (aus.tra.li.en′sis. N.L. nom. fem. adj. *australiensis* of Australia, where the isolate originated).

The following substrates are utilized: α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, Tween 40, *N*-acetyl-D-glucosamine, L-arabinose, cellobiose, *i*-erythritol, D-fructose, D-galactose, gentiobiose, α-D-glucose, *m*-inositol, α-D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, acetic acid, D-galacturonic acid, D-gluconic acid, α-hydroxybutyric acid, *p*-hydroxyphenyl acetic acid, itaconic acid, α-ketobutyric acid, propionic acid, quinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, γ-aminobutyric acid, inosine, putrescine, 2-aminoethanol, glycerol, arbutin, maltotriose, melezitose, palatinose, D-ribose, xylose, lactamide, pyruvic acid, *N*-acetyl-L-glutamic acid, adenosine, 2′-deoxyadenosine, fructose 6-phosphate, AMP and glucose 6-phosphate. Substrates not utilized by *Quadricoccus australiensis* include: *N*-acetyl-D-galactosamine, *cis*-aconitate, citrate, formate, D-galactonic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, L-histidine, L-pyroglutamic acid, D-serine, DL-carnitine, urocanic acid, uridine, thymidine, DL-α-glycerol phosphate, glucose 1-phosphate, inulin, mannan, Tween 80, *N*-acetyl-D-mannosamine, adonitol, amygdalin, arabitol, L-fucose, lactulose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, psicose, salicin, sedoheptulosan, stachyose, D-tagatose, turanose, xylitol, D-lactic acid methyl ester, L-lactic acid, DL-lactic acid, D-malic acid, L-malic acid, malonic acid, methyl pyruvate, monomethyl succinate, succinamic acid, 2,3-butanediol, TMP, UMP and DL-α-glycerol phosphate. Enzyme activities detected with API ZYM include esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. No alkaline phosphatase, lipase, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase are detected. Lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase activities are not detected with the Microbact 24E system and gelatin liquefaction is observed. Cells of *Quadricoccus australiensis* are H_2S-, indole- and Voges–Proskauer-negative but urease-positive. Growth occurs between 25 and 30 °C and pH values of 7·5 and 8·5. Natural habitat is activated sludge. Type strain is Ben 117^T (= NCIMB 13738^T = CIP 107055^T).

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