Defluvicoccus vanus gen. nov., sp. nov., a novel Gram-negative coccus/coccobacillus in the 'Alphaproteobacteria' from activated sludge

A. M. Maszenan,^{1†} R. J. Seviour,¹ B. K. C. Patel,² P. H. Janssen³ and J. Wanner⁴

Correspondence

R. J. Seviour

r.seviour@latrobe.edu.au

¹Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3550, Australia

²School of Biological and Biomedical Sciences, Faculty of Science and Technology, Griffith University, Nathan, Queensland 4111, Australia

³Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

⁴Department of Water Technology and Environmental Engineering, Prague Institute of Chemical Technology, Praha 6, Czech Republic

A novel Gram-negative coccus/coccobacillus, strain Ben 114^T, growing in tetrads, clusters or aggregates, was isolated from activated sludge by micromanipulation. 16S rRNA gene sequence analysis revealed that it belonged to the 'Alphaproteobacteria', with no close relatives among cultured bacterial isolates. On the basis of phylogenetic data, this organism is considered to belong to a new genus, *Defluvicoccus*, represented by the species *Defluvicoccus vanus* sp. nov., a name chosen because of the distinctive staining properties of this organism; only the cell wall stained strongly with a wide range of stains, giving the cell a hollow and empty appearance. No intracellular polyphosphate granules could be detected after staining, but poly- β -hydroxyalkanoate inclusions were detected using Nile blue A staining. Because of its taxonomic distance from its closest relatives among the 'Alphaproteobacteria', namely members of the genera *Azospirillum*, *Phaeospirillum*, *Rhodospirillum*, *Rhodocista*, *Magnetospirillum* and *Rhodospira*, *D. vanus* is considered to represent a new phylogenetic lineage within subgroup 1 of the 'Alphaproteobacteria', the *D. vanus* subgroup. The type strain is Ben 114^T (=NCIMB 13612^T = CIP 107350^T).

The application of molecular techniques to the study of activated sludge systems has revealed the presence of many previously uncultured bacteria (Seviour & Blackall, 1999; Loy *et al.*, 2002; Wagner & Loy, 2002). However, the techniques have not always provided a clearer explanation for how these systems might operate, a comment particularly relevant to processes of enhanced biological phosphorus removal (EBPR) (Loy *et al.*, 2002; Seviour *et al.*, 2003). Molecular studies on community structure of EBPR systems suggest that members of the *Actinobacteria* and 'Betaproteobacteria' closely related to members of the genus *Rhodocyclus* are probably responsible for phosphate removal

in some sludge plants (Hesseltmann *et al.*, 1999; Crocetti *et al.*, 2000; Zilles *et al.*, 2002; Seviour *et al.*, 2003). There is some evidence that other bacteria under certain conditions can out-compete the polyphosphate-accumulating organisms (PAO) during anaerobic substrate uptake, eventually leading to accumulation of glycogen-like carbohydrates instead of polyphosphate (polyP). Bacteria referred to as the 'glycogen-accumulating organisms' (GAO) are one such group (Seviour *et al.*, 2000; Crocetti *et al.*, 2002). They were originally called 'G-bacteria' because they dominated plants fed glucose (Cech & Hartman, 1993), and exist often as distinctive cocal cells in tetrads or clusters (Seviour *et al.*, 2000). Pure cultures of these tetrad-forming organisms (TFO) from different countries were described as separate species of a novel genus *Amaricoccus*, in subgroup 3 of the 'Alphaproteobacteria' (Maszenan *et al.*, 1997). Several other phylogenetically different TFO have also been isolated from activated sludge (Nakamura *et al.*, 1995; Yoshimi *et al.*, 1996; Maszenan *et al.*, 1999a, b, 2000). Some, like the actinobacterial *Tetrasphaera* species (Maszenan *et al.*, 2000) and the betaproteobacterium *Quadricoccus australiensis* (Maszenan *et al.*, 2002), appear to accumulate polyP in

[†]Present address: Environmental Engineering Research Centre, School of Civil and Environmental Engineering, Nanyang Technological University, Singapore 639798.

Abbreviations: EBPR, enhanced biological phosphorus removal; GAO, glycogen-accumulating organisms; PAO, polyphosphate-accumulating organisms; PHA, poly- β -hydroxyalkanoate; polyP, polyphosphate; TFO, tetrad-forming organisms.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ben 114^T is AF179678.

pure culture. However, their importance, if any, in EBPR plants remains unclear (Seviour *et al.*, 2003).

Here we describe a novel Gram-negative TFO (designated strain Ben 114^T) isolated from a sample of biomass from an EBPR activated sludge plant in the Czech Republic. 16S rRNA gene sequence analysis indicates that this organism is a member of the 'Alphaproteobacteria', but different from *Amaricoccus* species (Maszenan *et al.*, 1997) and *Q. australiensis* (Maszenan *et al.*, 2002) and with no known close relatives. We propose that it be placed in a new genus, *Defluvicoccus* gen. nov., as *Defluvicoccus vanus* sp. nov., representing a new phylogenetic lineage within subgroup 1 of the 'Alphaproteobacteria' (Woese *et al.*, 1984; Woese, 1987).

Strain Ben 114^T was isolated by micromanipulation from a sample of activated sludge biomass from an EBPR plant in Pilsen, Czech Republic, in August 1997 (Maszenan *et al.*, 1997). This EBPR plant had just started operation and good phosphate removal had not been achieved when the sample was obtained. Of many media routinely used to culture organisms from activated sludge that were tested (Maszenan *et al.*, 1997), freshly prepared GS medium (Williams & Unz, 1985) was the most successful in supporting growth of this organism from activated sludge. Purity of the cultures that grew was checked by light microscopic examination of single colonies, and only those consisting of distinctive TFO cells were then recovered for characterization. Strain Ben 114^T was stored at -80 °C (Maszenan *et al.*, 1997). The methods used for determining substrate utilization patterns, temperature and pH responses and biochemical characteristics were those described previously (Maszenan *et al.*, 1997), as were the staining methods used to identify polyP and poly- β -hydroxyalkanoate (PHA). Cells used to inoculate Biolog, API ZYM and Microbact 24E kits were grown on GS agar for 3 weeks at 25 °C. These characterizations were repeated in triplicate and always gave the same results. Genomic DNA G+C base composition was determined by HPLC with the method described by Janssen *et al.* (1996). Amplification of the 16S rRNA gene and its sequencing used the techniques detailed in Maszenan *et al.* (1997). Phylogenetic analysis after sequence alignment (Patel *et al.*, 1995) employed several methods described in the PHYLIP package (Felsenstein, 1993) including DNADIST (Jukes & Cantor, 1969) and neighbour-joining software. Trees were generated using TREECON after bootstrap and transversion analysis (van de Peer & de Wachter, 1993).

Strain Ben 114^T grew very slowly on GS agar, taking 2–3 weeks to produce visible mucoid beige colonies of <5 mm diameter. In GS broth dispersed growth was seen. Cocci/coccobacilli were irregular in size (1.5–4.0 μ m), usually growing in tetrads or clusters (Fig. 1), and the cells always showed Gram-negative staining. However, after staining with this and several other stains like methylene blue and toluidine blue, cells had a distinctive 'ghost-like' appearance (Fig. 1), which was much more apparent than was sometimes seen with *Amaricoccus* (Maszenan *et al.*,

Fig. 1. Light micrograph of strain Ben 114^T grown on GS medium showing clustered cells and unstained cell contents after polyP staining using the method of Rees *et al.* (1992). Bar, 10 μ m.

1997). No polyP was detected by staining, but an abundance of intracellular PHA granules was apparent after staining with Nile blue A (Rees *et al.*, 1992), when cells were grown aerobically on acetate, propionate or glucose (Maszenan *et al.*, 1997).

As mentioned above, Ben 114^T is a very slow-growing organism, and many of the biochemical tests used here produced negative results even after prolonged incubation, probably as a consequence of this. Ben 114^T is oxidase-negative, catalase-positive and weakly urease-positive. It failed to produce positive reactions with any of the Microbact 24E system tests (Oxoid), except that it was weakly positive for gelatin liquefaction. The following substrates were utilized with Biolog GN and GP systems: L-arabinose (weak), D-psicose, D-xylose (weak), α -DL-hydroxybutyrate, adonitol, D-lactic acid methyl ester, D-malate, methyl pyruvate, pyruvate, *N*-acetylglutamate, *N*-acetyl-D-glucosamine, i-erythritol, succinate, α -D-glucose, methyl β -D-glucoside, D-fructose (weak), monomethyl succinate (weak), methyl α -D-glucoside (weak), stachyose (weak), acetate, β -DL-hydroxybutyrate, lactamide (weak), L-lactate, L-malate, propionate, succinate, L-glutamate, L-threonine, quinate, L-aspartate, 3-methyl glucose, α -ketovalerate (weak), D-sorbitol (weak) and putrescine (weak).

With the API ZYM system, strain Ben 114^T was positive for the following enzyme activities: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. It was negative for valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, *N*-acetyl- β -glucosidase, α -mannosidase and α -fucosidase. It grew optimally at 25–30 °C and at a pH of 7.5–8.0. The DNA G+C composition was determined as 66 mol% (Table 1).

Table 1. Comparative phenotypic characteristics of strain Ben 114^T and selected genera of subgroup 1 of the '*Alphaproteobacteria*'

Data for reference taxa were taken from Falk *et al.* (1985), Reinhold *et al.* (1987), Tarrand *et al.* (1978) and Krieg & Döbereiner (1984) (*Azospirillum*), Imhoff *et al.* (1998) (*Rhodospirillum*), Favinger *et al.* (1989) and Kawasaki *et al.* (1992) (*Rhodocista*), Pfennig *et al.* (1997) (*Rhodospira*) and Maratea & Blakemore (1981), Schleifer *et al.* (1991) and Burgess *et al.* (1993) (*Magnetospirillum*). +, Positive; –, negative; ND, not determined; NA, not available.

Characteristic	Strain Ben 114 ^T	<i>Azospirillum</i>	<i>Rhodospirillum</i>	<i>Rhodocista</i>	<i>Rhodospira</i>	<i>Magnetospirillum</i>
Morphology (cell size, µm)	Cocci/coccobacilli in tetrads and clusters (1.5–4.5)	Straight to slightly curved rods (0.7–1.4)	Spiral shaped cells (0.8–1.0)	Spirilloid (1.0–2.0), vibrioid cells, cyst-forming bacteria	Spirilloid with one S-turn, vibrioid (0.6–0.8)	Helical cells (0.2–0.7)*
Polysaccharide granules	ND	NA	+	+	NA	NA
Motility	Non-motile	Motile	Motile	Motile	Motile	Motile
Flagella	Absent	Single, polar in liquid medium, polytrichous on solid media	Bipolar tufts	Polar flagella	Bipolar flagellar tufts	Single bipolar flagellum
DNA G+C content (mol%)	66	67–71	63–66	69–70	65.7	64–71*
Oxidase	–	+	+	+	NA	+
Catalase	+	+	+	+	ND	+
Habitat	Activated sludge biomass	Soils and roots of cereal crops, grasses and tuber plants	Mud or water	Water sample (temperature 55 °C) at the edge of source pool, Thermopolis Hot Springs, WY, USA	Microbial mats from Great Sippewissett salt marsh, Cape Cod, MA, USA	Freshwater sources including distilled water, ditch and canal water and sewage
O ₂ requirement	Aerobic	Aerobic	Anaerobic in light, aerobic to microaerophilic in dark	Aerobic and microaerophilic	Strictly anoxic	Microaerophilic*
Optimum temperature (°C)	25–30	34–37†	NA	39–45	25–30 (at 2 % NaCl), 20–35 (at 0.5–5.0 % NaCl)	30
Optimum pH	7.5–8.0	5.8–6.8†	NA	6.8	7.3–7.5 (at 2 % NaCl), 7.0–7.8 (at 0.5–5.0 % NaCl)	NA
Urease	+	+	NA	NA	NA	–
Nitrate reduction	+	+	–	NA	NA	+
Gelatin hydrolysis	+	–	NA	NA	NA	–

*Strains AMB-1 and MGT-1 (Burgess *et al.*, 1993) and *Magnetospirillum magnetotacticum* (Maratea & Blakemore, 1981) are catalase- and oxidase-negative and the former two are facultative anaerobes. Data from Schleifer *et al.* (1991).

†Except for *Azospirillum halopraeferans*, which has an optimal temperature of 41 °C and optimal pH of 8.0.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence analysis of strain Ben 114^T showing its relationship to other members of subgroup 1 of the 'Alphaproteobacteria', constructed using the neighbour-joining method. All sequences were obtained from the Ribosomal Database Project except for *Rhodospira trueperi*, which was extracted from GenBank. Bootstrap values from 100 replicates are indicated at branch points. Bar, 10 nucleotide substitutions per 100 nucleotides.

On the basis of 1212 nucleotides, phylogenetic analysis (Fig. 2) revealed that Ben 114^T is a member of subgroup 1 of the 'Alphaproteobacteria', with no close relatives among recognized organisms in this class. This phylogeny was robust, as supported by high bootstrap values and stability after transversion analysis. The neighbour-joining tree shows that strain Ben 114^T does not fall within the currently proposed alphaproteobacterial groupings, i.e. the *Rhodospirillum rubrum* (Molisch, 1907; Imhoff *et al.*, 1998), *Phaeospirillum fulvum* (Imhoff *et al.*, 1998) and *Magnetospirillum gryphiswaldense* groupings (Maidak *et al.*, 1999). Its 16S rRNA gene sequence showed similarities of up to 87.5% to that of the *Rhodospirillum rubrum* group, up to 88.5% to the *P. fulvum* group, 88.2% to *M. gryphiswaldense* (Schleifer *et al.*, 1991; Burgess *et al.*, 1993), 88.5% to *Magnetospirillum magnetotacticum* (Maratea & Blakemore, 1981; Eden *et al.*, 1991; Schleifer *et al.*, 1991; Burgess *et al.*, 1993) and 89% to *Rhodocista centenaria* (Favinger *et al.*, 1989; Kawasaki *et al.*, 1992). A comparison of the inferred 16S rRNA gene sequence signature nucleotides between members of subgroup 1 of the 'Alphaproteobacteria' supports the view that Ben 114^T is not closely related to any of them. For example, only strain Ben 114^T possessed U-A, G-U, U-G and G-U at *Escherichia coli* positions 333–366, 425–434, 436–441 and 1218–1223, respectively (Table 2).

The isolation and characterization of strain Ben 114^T has extended the number and hence the extent of the phylogenetic diversity of the TFO reported in activated sludge systems (Seviour *et al.*, 2000). Earlier work in our laboratories (Maszenan *et al.*, 1999a, b, 2000) and elsewhere (Nakamura *et al.*, 1995; Yoshimi *et al.*, 1996; Shintani *et al.*, 2000) has revealed the presence of several novel Gram-positive cocci in this complex ecosystem (Seviour *et al.*, 2000). Although their roles and functions there are not well understood, it is possible from their pure culture behaviour

that some may participate in EBPR (Nakamura *et al.*, 1995; Maszenan *et al.*, 1999a, b, 2000).

Nielsen *et al.* (1999), Crocetti *et al.* (2002) and Kong *et al.* (2002) all reported novel Gram-negative cocci in activated sludge with poor EBPR capacity, which were identified after fluorescence *in situ* hybridization as members of the 'Gammaproteobacteria'. Strain Ben 114^T is clearly phylogenetically different from these isolates, but, like the *Amaricoccus* isolates (Maszenan *et al.*, 1997) and these gammaproteobacteria, it appears unable to accumulate polyP. However, its ability to synthesize PHA aerobically in pure culture is not consistent with the phenotype of a GAO (Hesselmann *et al.*, 1999; Crocetti *et al.*, 2002; Seviour *et al.*, 2003).

Phylogenetic analysis of the 16S rRNA gene sequence of Ben 114^T (Fig. 2) shows that it is a novel, deeply branching member of the 'Alphaproteobacteria' with no recognized close relatives, a proposal supported based on differences in the phenotypic characteristics of these taxa (Table 1) and inferred 16S rRNA gene sequence signature nucleotides (Table 2). The data presented support the view that strain Ben 114^T is representative of a new genus and species, for which the name *Defluvicoccus vanus* gen. nov., sp. nov. is proposed. Because of its low level of similarity to recognized members of this division, it is proposed that *Defluvicoccus* is a member of a novel phylogenetic lineage within subgroup 1 of the 'Alphaproteobacteria' (Woese *et al.*, 1984; Woese, 1987), separate from both the *P. fulvum* and the *Rhodospirillum rubrum* lineages (Imhoff *et al.*, 1998).

Reclassification of the spiral-shaped, phototrophic, purple, non-sulfur bacteria of the 'Alphaproteobacteria' by Imhoff *et al.* (1998) has led to the description of several novel species and genera. The signature 16S rRNA gene sequence nucleotides suggested by Woese (1987) to delineate the

Table 2. Comparative DNA signature nucleotides for strain Ben 114^T and related genera from subgroup 1 of the 'Alphaproteobacteria'

Positions are according to the *E. coli* numbering system.

Position	Azospirillum	Magnetospirillum	Phaeospirillum	Rhodocista	Rhodovibrio	Rhodospira	Rhodothalassium	Rhodospirillum	Roseospira	Ben 114 ^T
333–366	C–A	C–A	C–A	C–A	C–C	C–A	C–C	C–A	C–A	T–A
425–434	A–C, G–C	G–C	G–C	G–C	G–C	G–C	G–C	G–C	G–C	G–T
436–441	C–A, C–G	C–G, C–A	C–G	C–G	N–G	C–A	C–A	C–G	C–A	T–G
582–586	T–T, U–C	C–C	C–C	T–C	T–C	T–C	T–C	T–C	T–C	C–C
933–963	G–C	G–C	G–C	G–C	G–C	C–C	G–C	G–C	G–C	G–T
990–997	C–T	C–T	C–T	C–T	C–T	C–T	C–T	C–T	C–T	T–T
1046–1072	A–G	A–G	A–G	A–G	A–G	A–G	A–G	A–G	A–G	A–T
1092–1099	A–G	A–G	A–G	A–G	A–G	A–G	A–G	A–G	A–G	C–G
1218–1223	C–C, T–C	C–C	C–C	T–C	C–C	C–C	C–C	T–C, C–C	C–C	G–T

'Alphaproteobacteria' are generally still applicable and strain Ben 114^T possesses all of these (data not shown). However, a few modifications to the scheme of Woese (1987) are required in order to accommodate these newly described taxa. For example, at position 812 *Roseospira* (Kompantseva & Gorlenko, 1984; Imhoff *et al.*, 1998) has U instead of G, while at position 933 *Rhodospira* (Pfennig *et al.*, 1997) possesses C instead of G. In addition, at position 822 *Rhodothalassium* (Drews, 1981; Imhoff *et al.*, 1998) has G instead of A or U, and at position 823 *Rhodocista* (Favinger *et al.*, 1989; Kawasaki *et al.*, 1992) has C instead of G or A.

Description of *Defluvicoccus* gen. nov.

Defluvicoccus [De.flu.vi.coc'cus. L. neut. n. *defluvium* sewage; N.L. (Gr. derived) masc. n. *coccus* berry (spherical microbe); N.L. masc. n. *Defluvicoccus* a coccus from sewage].

Produces mucoid beige colonies <5 mm in diameter after 3–4 weeks incubation at 25 °C on GS medium. Gram-negative, chemoheterotrophic, non-spore-forming, non-motile and aerobic cocci/coccobacilli (mean cell diameter 1.5–4.0 µm), which are usually arranged in clusters or tetrads. Cells stain very faintly and appear empty after staining. Oxidase-negative and catalase-positive. The type species is *Defluvicoccus vanus*.

Description of *Defluvicoccus vanus* sp. nov.

Defluvicoccus vanus (va'nus. L. masc. adj. *vanus* empty, idle, pertaining to its staining behaviour).

Shows the following properties in addition to those included in the genus description. No polyP granules can be detected in axenic cultures, although glucose-, acetate- and propionate-grown cells contain PHA granules. The following substrates are utilized: L-arabinose, D-psicose, D-xylose, α-DL-hydroxybutyrate, adonitol, D-lactic acid methyl ester, D-malate, methyl pyruvate, pyruvate, N-acetylglutamate, i-erythritol, succinate, α-ketoglutarate, D-sorbitol, putrescine, methyl α-D-mannoside, stachyose, acetate, β-DL-hydroxybutyrate, lactamide, L-lactate, L-malate, propionate, L-glutamate, L-threonine, quinate, L-aspartate, D-fructose, α-D-glucose, 3-methyl glucose, methyl β-D-glucoside and monomethyl succinate. Positive for the following enzyme activities as detected with the API ZYM system: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Growth occurs at temperatures between 20 and 30 °C and at pH values between 5.0 and 8.5. Weakly positive for urease and gelatin liquefaction. Does not produce H₂S, indole or acetoin. The DNA G + C content is 66 mol%.

The type strain, Ben 114^T (=NCIMB 13612^T = CIP 107350^T), was isolated from activated sludge.

Acknowledgements

A.M.M. was supported by an LUPRS and LUOPRS scholarship. A La Trobe University Central Grant and an ARC Large Grant to R. J. S. supported this work. We thank Professor H. G. Trüper for his assistance with naming of the organism.

References

- Burgess, J. G., Kawaguchi, R., Sakaguchi, T., Thornhill, R. H. & Matsunaga, T. (1993). Evolutionary relationships among *Magnetospirillum* strains inferred from phylogenetic analysis of 16S rDNA sequences. *J Bacteriol* **175**, 6689–6694.
- Cech, J. S. & Hartman, P. (1993). Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. *Water Res* **27**, 1219–1225.
- Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D. & Blackall, L. L. (2000). Identification of polyphosphate-accumulating organisms and design of 16S rRNA directed probes for their detection and quantitation. *Appl Environ Microbiol* **66**, 1175–1182.
- Crocetti, G. R., Banfield, J. F., Keller, J., Bond, P. L. & Blackall, L. L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology* **148**, 3353–3364.
- Drews, G. (1981). *Rhodospirillum salexigens*, spec. nov., an obligately halophilic phototrophic bacterium. *Arch Microbiol* **130**, 325–327.
- Eden, P. A., Schmidt, T. M., Blakemore, R. P. & Pace, N. R. (1991). Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *Int J Syst Bacteriol* **41**, 324–325.
- Falk, E. C., Döbereiner, J., Johnson, J. L. & Krieg, N. R. (1985). Deoxyribonucleic acid homology of *Azospirillum amazonense* Magalhães et al. 1984 and emendation of the description of the genus *Azospirillum*. *Int J Syst Bacteriol* **35**, 117–118.
- Favinger, J., Stadtwald, R. & Gest, H. (1989). *Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. *Antonie van Leeuwenhoek* **55**, 291–296.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package), version 3.51c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Hesselmann, R. P. X., Werlen, C., Hahn, D., van der Meer, J. R. & Zehnder, A. J. B. (1999). Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. *Syst Appl Microbiol* **22**, 454–465.
- Imhoff, J. F., Petri, R. & Süling, J. (1998). Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the α -Proteobacteria: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* comb. nov. to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *Int J Syst Bacteriol* **48**, 793–798.
- Janssen, P. H., Liesack, W., Kluge, C., Seeliger, S., Schink, B. & Harfoot, C. G. (1996). Sodium-dependent succinate decarboxylation by a new anaerobic bacterium belonging to the genus *Peptostreptococcus*. *Antonie van Leeuwenhoek* **70**, 11–20.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kawasaki, H., Hoshino, Y., Kuraishi, H. & Yamasato, K. (1992). *Rhodocista centenaria* gen. nov., sp. nov., a cyst-forming anoxygenic photosynthetic bacterium and its phylogenetic position in the Proteobacteria alpha group. *J Gen Appl Microbiol* **38**, 541–551.
- Kompantseva, E. I. & Gorlenko, V. M. (1984). A new species of moderately halophilic purple bacterium *Rhodospirillum mediosalinum* sp. nov. *Mikrobiologiya* **53**, 954–961 (in Russian).
- Kong, Y., Ong, S. L., Ng, W. J. & Liu, W. T. (2002). Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic–aerobic activated sludge processes. *Environ Microbiol* **4**, 753–757.
- Krieg, N. R. & Döbereiner, J. (1984). Genus *Azospirillum* Tarrand, Krieg and Döbereiner 1979, 79^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 94–104. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Loy, A., Daims, H. & Wagner, M. (2002). Activated sludge – molecular techniques for determining community composition. In *Encyclopaedia for Environmental Microbiology*, pp. 26–43. Edited by G. Bitton. New York: Wiley Scientific.
- Maidak, B. L., Cole, J. R., Parker, C. T., Jr & 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.
- Maratea, D. & Blakemore, R. P. (1981). *Aquaspirillum magnetotacticum* sp. nov., a magnetic spirillum. *Int J Syst Bacteriol* **31**, 452–455.
- Maszenan, A. M., Seviour, R. J., Patel, B. K. C., Rees, G. N. & McDougall, B. M. (1997). *Amaricoccus* gen. nov., a gram-negative coccus occurring in regular packages or tetrads, isolated from activated sludge biomass, and descriptions of *Amaricoccus veronensis* sp. nov., *Amaricoccus tamworthensis* sp. nov., *Amaricoccus macauensis* sp. nov., and *Amaricoccus kaplicensis* sp. nov. *Int J Syst Bacteriol* **47**, 727–734.
- Maszenan, A. M., Seviour, R. J., Patel, B. K. C., Schumann, P. & Rees, G. N. (1999a). *Tessaracoccus bendigoensis* gen. nov., sp. nov., a Gram-positive coccus occurring in regular packages or tetrads, isolated from activated sludge biomass. *Int J Syst Bacteriol* **49**, 459–468.
- Maszenan, A. M., Seviour, R. J., Patel, B. K. C., Schumann, P., Burghardt, J., Webb, R. I., Soddell, J. A. & Rees, G. N. (1999b). *Friedmanniella spumicola* sp. nov. and *Friedmanniella capsulata* sp. nov. from activated sludge foam: Gram-positive cocci that grow in aggregates of repeating groups of cocci. *Int J Syst Bacteriol* **49**, 1667–1680.
- Maszenan, A. M., Seviour, R. J., Patel, B. K. C., Schumann, P., Burghardt, J., Tokiwa, Y. & Stratton, H. M. (2000). 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. *Int J Syst Evol Microbiol* **50**, 593–603.
- Maszenan, A. M., Seviour, R. J., Patel, B. K. C. & Schumann, P. (2002). *Quadricoccus australiensis* gen. nov., sp. nov., a β -proteobacterium from activated sludge biomass. *Int J Syst Evol Microbiol* **52**, 223–228.
- Molisch, H. (1907). *Die Purpurbakterien nach neuen Untersuchungen*. Jena: G. Fischer (in German).
- Nakamura, K., Hiraishi, A., Yoshimi, Y., Kawaharasaki, M., Masuda, K. & Kamagata, Y. (1995). *Micrococcus phosphovorans* gen. nov., sp. nov., a new gram-positive polyphosphate-accumulating bacterium isolated from activated sludge. *Int J Syst Bacteriol* **45**, 17–22.

- Nielsen, A. T., Liu, W.-T., Filipe, C., Grady, L., Jr, Molin, S. & Stahl, D. A. (1999). Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. *Appl Environ Microbiol* **65**, 1251–1258.
- Patel, B. K. C., Andrews, K. T., Ollivier, B., Mah, R. A. & Garcia, J. L. (1995). Reevaluating the classification of *Halobacteriodes* and *Haloanaerobacter* species based on sequence comparisons of the 16S ribosomal RNA gene. *FEMS Microbiol Lett* **134**, 115–119.
- Pfennig, N., Lünsdorf, H., Süling, J. & Imhoff, J. F. (1997). *Rhodospira trueperi* gen. nov., spec. nov., a new phototrophic proteobacterium of the alpha group. *Arch Microbiol* **168**, 39–45.
- Rees, G. N., Vasiliadis, G., May, J. W. & Bayly, R. C. (1992). Differentiation of polyphosphate and poly- β -hydroxybutyrate granules in an *Acinetobacter* sp. isolated from activated sludge. *FEMS Microbiol Lett* **94**, 171–174.
- Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S. & De Ley, J. (1987). *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). *Int J Syst Bacteriol* **37**, 43–51.
- Schleifer, K.-H., Schöler, D., Spring, S., Weizenegger, M., Amann, R., Ludwig, W. & Köhler, M. (1991). The genus *Magnetospirillum* gen. nov.: description of *Magnetospirillum gryphiswaldense* sp. nov. and transfer of *Aquaspirillum magnetotacticum* to *Magnetospirillum magnetotacticum* comb. nov. *Syst Appl Microbiol* **14**, 379–385.
- Seviour, R. J. & Blackall, L. L. (1999). *Microbiology of Activated Sludge*. Dordrecht: Kluwer Academic.
- Seviour, R. J., Maszenan, A. M., Soddell, J. A., Tandoi, V., Patel, B. K. C., Kong, Y. & Schumann, P. (2000). Microbiology of the 'G-bacteria' in activated sludge. *Environ Microbiol* **2**, 581–593.
- Seviour, R. J., Mino, T. & Onuki, M. (2003). The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol Rev* **27**, 99–127.
- Shintani, T., Liu, W.-T., Hanada, S., Kamagata, Y., Miyaoka, S., Suzuki, Y. & Nakamura, K. (2000). *Micropruina glycogenica* gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. *Int J Syst Evol Microbiol* **50**, 201–207.
- Tarrand, J. J., Krieg, N. R. & Döbereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol* **24**, 967–980.
- van de Peer, Y. & de Wachter, R. (1993). TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**, 177–182.
- Wagner, M. & Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. *Curr Opin Biotechnol* **13**, 218–227.
- Williams, T. M. & Unz, R. F. (1985). Isolation and characterization of filamentous bacteria present in bulking activated sludge. *Appl Environ Microbiol* **22**, 273–282.
- Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.
- Woese, C. R., Stackebrandt, E., Weisburg, W. G. & 8 other authors (1984). The phylogeny of purple bacteria: the alpha subdivision. *Syst Appl Microbiol* **5**, 315–326.
- Yoshimi, Y., Hiraishi, A. & Nakamura, K. (1996). Isolation and characterization of *Microsphaera multipartita* gen. nov., sp. nov., a polysaccharide-accumulating gram-positive bacterium from activated sludge. *Int J Syst Bacteriol* **46**, 519–525.
- Zilles, J. L., Peccia, J., Kim, M.-W., Hung, C.-H. & Noguera, D. R. (2002). Involvement of *Rhodocyclus*-related organisms in phosphorus removal in full-scale wastewater treatment plants. *Appl Environ Microbiol* **68**, 2763–2769.