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

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A novel Gram-negative coccus/coccobacillus, strain Ben 114\(^1\), 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-\(\beta\)-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\(^1\) (= 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 Rhodococcus are probably responsible for phosphate removal in some sludge plants (Hesselmann 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 coccal 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

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ben 114\(^1\) 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 Amarinococcus 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-\(\beta\)-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 \(\mu\)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 Amarinococcus (Maszenan et al., 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-xylene (weak), \(\alpha\)-DL-hydroxybutyrate, adonitol, d-lactic acid methyl ester, d-malate, methyl pyruvate, pyruvate, N-acetylglutamate, N-acetyl-D-glucosamine, i-erythritol, succinate, \(\alpha\)-D-glucose, methyl \(\beta\)-D-glucoside, D-fructose (weak), monomethyl succinate (weak), methyl \(\alpha\)-D-glucoside (weak), stachyose (weak), acetate, \(\beta\)-DL-hydroxybutyrate, lactamide (weak), L-lactate, L-malate, propionate, succinate, \(\alpha\)-glutamate, l-threonine, quinate, L-aspartate, 3-methyl glucose, \(\alpha\)-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, \(\alpha\)-galactosidase, \(\beta\)-galactosidase, \(N\)-acetyl-\(\beta\)-glucosidase, \(\alpha\)-mannosidase and \(\alpha\)-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’


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain Ben 114^T</th>
<th>Azospirillum</th>
<th>Rhodospirillum</th>
<th>Rhodocista</th>
<th>Rhodospira</th>
<th>Magnetospirillum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Cocci/coccobacilli in tetrads and clusters (1·5−4·5)</td>
<td>Straight to slightly curved rods (0·7−1·4)</td>
<td>Spiral shaped cells (0·8−1·0)</td>
<td>Spirilloid (1·0−2·0), vibrioid cells, cyst-forming bacteria</td>
<td>Spirilloid with one S-turn, vibrioid (0·6−0·8)</td>
<td>Helical cells (0·2−0·7)*</td>
</tr>
<tr>
<td>Polysaccharide granules</td>
<td>ND</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Motility</td>
<td>Non-motile</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Flagella</td>
<td>Absent</td>
<td>Single, polar in liquid medium, polytrichous on solid media</td>
<td>Bipolar tufts</td>
<td>Polar flagella</td>
<td>Bipolar flagellar tufts</td>
<td>Single bipolar flagellum</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66</td>
<td>67−71</td>
<td>63−66</td>
<td>69−70</td>
<td>65·7</td>
<td>64−71*</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>+*</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>Habitat</td>
<td>Activated sludge biomass</td>
<td>Soils and roots of cereal crops, grasses and tuber plants</td>
<td>Mud or water</td>
<td>Water sample (temperature 55 °C) at the edge of source pool, Thermopolis Hot Springs, WY, USA</td>
<td>Microbial mats from Great Sippewissett salt marsh, Cape Cod, MA, USA</td>
<td>Freshwater sources including distilled water, ditch and canal water and sewage</td>
</tr>
<tr>
<td>O₂ requirement</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Anaerobic in light, aerobic to microaerophilic in dark</td>
<td>Aerobic and microaerophilic</td>
<td>Strictly anoxic</td>
<td>Microaerophilic*</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>25−30</td>
<td>34−37†</td>
<td>NA</td>
<td>39−45</td>
<td>25−30 (at 2% NaCl), 20−35 (at 0·5−5·0% NaCl)</td>
<td>30</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7·5−8·0</td>
<td>5·8−6·8†</td>
<td>NA</td>
<td>6·8</td>
<td>7·3−7·5 (at 2% NaCl), 7·0−7·8 (at 0·5−5·0% NaCl)</td>
<td>NA</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+ (weak)</td>
<td>+</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+ (weak)</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
</tr>
</tbody>
</table>

*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.
On the basis of 1212 nucleotides, phylogenetic analysis (Fig. 2) revealed that Ben 114\textsuperscript{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\textsuperscript{T} does not fall within the currently proposed alphaproteobacterial groupings, i.e. the Rhodospirillum rubrum (Molisch, 1907; Imhoff et al., 1998), Phaeospiroplum 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\textsuperscript{T} is not closely related to any of them. For example, only strain Ben 114\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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
‘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.coccus. 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-acetylglутamate, i-erythritol, succinate, α-ketovalerate, d-sorbitol, putrescine, methyl α-D-mannoside, stachyose, acetate, β-DL-hydroxybutyrate, lactamide, L-lactate, L-malate, propionate, L-glutamate, L-threonine, quinate, 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

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References


