Brevundimonas aveniformis sp. nov., a stalked species isolated from activated sludge

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A Gram-negative, rod-like, stalk-producing bacterium, designated strain EMB102^T, was isolated from activated sludge that performed enhanced biological phosphorus removal in a sequencing batch reactor. Cells without stalks were motile with single polar flagella, but cells that did produce stalks were non-motile and lacked polar flagella. Growth of strain EMB102^T was observed at temperatures between 15 and 35 °C (optimum, 30 °C) and between pH 6.0 and 9.0 (optimum, pH 7.5–8.5). The predominant fatty acids of strain EMB102^T were C_{18:1}ω7c, C_{16:0} and C_{15:0}. The predominant polar lipid was phosphatidylglycerol. The G + C content of the genomic DNA was 64.1 mol% and the major quinone was Q-10. Comparative 16S rRNA gene sequence analyses showed that strain EMB102^T formed a distinct phyletic lineage within the genus *Brevundimonas*. The levels of 16S rRNA gene sequence similarity between the type strains of *Brevundimonas* species ranged from 95.8 to 97.5 %. DNA–DNA relatedness levels between the EMB102^T and closely related *Brevundimonas* species were below 15.0 %. On the basis of chemotaxonomic data and molecular properties, strain EMB102^T represents a novel species within the genus *Brevundimonas*, for which the name *Brevundimonas aveniformis* sp. nov. is proposed. The type strain is EMB102^T (=KCTC 12609^T=DSM 17977^T).

Activated sludge processes involving cyclical changes between anaerobic and aerobic conditions have been employed to remove phosphate from wastewater, and are increasingly used to reduce eutrophication in lakes (Mino *et al.*, 1987; Jeon & Park, 2000). Insight into the bacterial community responsible for phosphorus removal is a prerequisite for an understanding of the enhanced biological phosphorus removal (EBPR) mechanism and for controlling the EBPR processes. *Pseudomonas diminuta* and *Pseudomonas vesicularis*, first described by Leifson & Hugh (1954), were reclassified into a novel genus, *Brevundimonas* (in the *Alphaproteobacteria*) with *Brevundimonas diminuta* by Segers *et al.* (1994). Later, several *Caulobacter* species and subspecies were transferred to the genus *Brevundimonas*, and novel *Brevundimonas* species isolated from diverse habitats, especially marine habitats, were described (Abraham *et al.*, 1999; Fritz *et al.*, 2005; Li *et al.*,

2004). In this study, we describe the taxonomic characterization of a novel species, isolated from activated sludge that was able to perform EBPR, and we propose a novel species of the genus *Brevundimonas*.

Strain EMB102^T was isolated from activated sludge performing EBPR in a laboratory-scale sequencing batch reactor. Sodium acetate was supplied as a sole carbon source and the operation of the sequencing batch reactor was as described elsewhere (Jeon *et al.*, 2003). The sludge sample was serially diluted with 1 % (w/v) saline solution and spread on R2A agar (Difco) and incubated at 20 °C for 7 days. Subcultivation was conducted on R2A agar at 30 °C for 3 days.

Physiological characteristics of strain EMB102^T and type strains of members of the genus *Brevundimonas* were examined; the isolate was grown on R2A medium at different temperatures and pH values. R2A media with different pHs were prepared as described previously (Gomori, 1955). Gram staining was performed using a bioMérieux Gram stain kit according to the instructions of the manufacturer. Oxidase activity was tested by assessing the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was evaluated by observing

Abbreviation: EBPR, enhanced biological phosphorus removal.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB102^T is DQ372984.

The cellular fatty acid compositions and substrate-assimilation data for strain EMB102^T and some related *Brevundimonas* species are available in supplementary tables available with the online version of this paper.

the production of oxygen bubbles in a 3% (v/v) aqueous hydrogen peroxide solution. Cell morphology and motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) at different growth stages, as described by Jeon *et al.* (2005). Hydrolysis of casein, gelatin, Tweens 80 and 20, aesculin, urea, tyrosine and starch was investigated on R2A agar after 7 days incubation, as described previously (Lanyi, 1987; Gerhardt *et al.*, 1994). Growth at various NaCl concentrations was investigated in 10-fold-diluted trypticase soy broth (Difco). Nitrate reduction was determined according to the method of Lanyi (1987), and acid production from carbohydrates was tested as described by Leifson (1963). Oxidation of various substrates was determined by using the Biolog GN2 MicroPlate assay as recommended by the manufacturer, and additional enzyme activities and biochemical features were determined by using API kits (API ZYM and API 20E) at 30 °C as recommended by the manufacturer (bioMérieux). Growth of strain EMB102^T was obtained at temperatures between 15 and 35 °C, the optimum growth temperature being 30 °C. The strain grew at pH 6.0–9.0, the optimum being at pH 7.5–8.5. Cells of strain EMB102^T were found to be Gram-negative and oxidase- and catalase-positive. During the early growth stage, the cells were motile by means of single polar flagella, but they gradually lost their flagella and each produced a stalk at the flagellated pole: this is the typical lifestyle of some species of the genera *Caulobacter* and *Brevundimonas* (Fig. 1). The number of stalked cells lacking motility increased with increasing cultivation time. The cylindrical body of the cell was approximately 0.3–0.4 µm in diameter and 1.0–2.0 µm in length. Anaerobic growth was not observed at 7 days cultivation on R2A agar at 30 °C, but weak growth was observed after 16 days. The strain reduced nitrate to nitrite and produced nitrogen gas. Other phenotypic features of strain EMB102^T and type strains of *Brevundimonas* species are presented in Table 1 and in the description of the novel species. The oxidation results for various substrates tested with the Biolog GN2 MicroPlate system are shown in

Fig. 1. Transmission electron micrographs showing the general morphology of negatively stained cells of strain EMB102^T after growth at 30 °C in R2A broth. (a) Motile cell with a flagellum; (b) non-motile stalked cells without flagella. Bars, 1 µm.

Supplementary Table S1 (available with the online version of this paper). Some of these results are in accordance with the characteristics of members of the genus *Brevundimonas*, whereas some others allow the differentiation of strain EMB102^T from closely related species of this genus (Table 1).

For analysis of fatty acid methyl esters, cells of strain EMB102^T and type strains of *Brevundimonas* species were harvested from agar plates after incubation for 3 days on LMG medium no. 221 at the relevant optimal temperature. Analysis of the fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Analyses of isoprenoid quinones and polar lipids were carried out using the methods described by Komagata & Suzuki (1987) and Batrakov *et al.* (1997), respectively. The DNA G+C content of strain EMB102^T was determined using HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE, GROM) according to the method of Tamaoka & Komagata (1984). The major cellular fatty acids present were C_{18:1}ω7c, C_{16:0} and C_{15:0}. The main hydroxy fatty acid was iso-C_{17:0} 3-OH on LMG medium no. 221, and the fatty acid profile was generally similar to those of other *Brevundimonas* species; however, the proportions of some fatty acids, such as C_{18:1} 11-methyl ω7c, C_{17:1}ω7c and C_{19:0} cyclo ω8c were different (see Supplementary Table S2 available with the online version of this paper). The predominant polar lipid of strain EMB102^T was phosphatidylglycerol, the major respiratory lipoquinone was Q-10 and the DNA G+C content was 64.1 mol%. The major fatty acids, the major lipoquinone, the polar lipid profile and the G+C content of strain EMB102^T are in accordance with those of members of the genus *Brevundimonas* (Abraham *et al.*, 1999; Li *et al.*, 2004; Fritz *et al.*, 2005; Yoon *et al.*, 2006).

The sequencing and assembly of the 16S rRNA gene were carried out as described by Lane (1991). The resultant 16S rRNA gene sequence (1403 nt) of strain EMB102^T was compared with those available from GenBank by using the BLAST program (www.ncbi.nlm.nih.gov/blast/) to determine an approximate phylogenetic affiliation; the gene sequences were aligned with those of closely related species by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were constructed using three different methods, i.e. the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms; these methods are available in PHYLIP software, version 3.6 (Felsenstein, 2002). Values for sequence similarity between the isolate and related members of the genus *Brevundimonas* were computed using SIMILARITY MATRIX, version 1.1 (Ribosomal Database Project II; <http://35.8.164.52/html/>; Cole *et al.*, 2003). A bootstrap analysis was performed according to the algorithm of the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP package. DNA–DNA hybridization experiments were carried out to evaluate the genomic DNA relatedness between strain EMB102^T and *Brevundimonas nasdae*

Table 1. Phenotypic characteristics of strain EMB102^T and some related *Brevundimonas* species

Taxa: 1, strain EMB102^T; 2, *Brevundimonas diminuta*; 3, *Brevundimonas kwangchunensis*; 4, *Brevundimonas alba*; 5, *Brevundimonas aurantiaca*; 6, *Brevundimonas intermedia*; 7, *Brevundimonas nasdae*; 8, *Brevundimonas vesicularis*. Symbols: +, positive; –, negative; w, weakly positive reaction. Data in parentheses are for type strains. Type strains of all species were positive for oxidase and catalase (this study).

Characteristic	1*	2	3	4*	5	6	7	8
Urease*	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
Arginine dihydrolase	(–)	–	–	(–)	(–)	(–)	–	–
β-Galactosidase	(–)	–	–	(–)	(–)	(+)	+	–
Nitrate reduction*	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
Hydrolysis of:*								
Aesculin	(–)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
Gelatin	(+)	(–)	(–)	(–)	(+)	(–)	(–)	(–)
Starch	(–)	(–)	(–)	(–)	(+)	(+)	(–)	(–)
Tween 80	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)
Utilization of:*								
D-Cellobiose	(–)	(–)	(+)	(+)	(+)	(+)	(–)	(+)
D-Fructose	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)
D-Galactose	(–)	(–)	(–)	(+)	(–)	(+)	(–)	(+)
α-D-Glucose	(–)	(–)	(+)	(+)	(+)	(+)	(–)	(+)
α-D-Lactose	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(w)
Maltose	(–)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
D-Mannose	(–)	(–)	(+)	(–)	(+)	(+)	(+)	(–)
D-Trehalose	(–)	(–)	(+)	(w)	(+)	(+)	(–)	(+)
Turanose	(–)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
Acetic acid	(–)	(+)	(+)	(w)	(–)	(+)	(–)	(+)
DL-Lactic acid	(–)	(–)	(+)	(–)	(+)	(+)	(+)	(+)
Succinic acid	(w)	(+)	(+)	(+)	(+)	(+)	(–)	(+)
L-Aspartic acid	(+)	(+)	(+)	(+)	(+)	(+)	(–)	(+)
L-Histidine	(–)	(+)	(–)	(–)	(–)	(–)	(–)	(–)
L-Leucine	(–)	(+)	(–)	(–)	(–)	(–)	(–)	(–)
L-Proline	(+)	(+)	(+)	(+)	(–)	(+)	(w)	(+)
α-Glucose 1-phosphate	(–)	(+)	(–)	(–)	(+)	(–)	(–)	(+)
Glucose 6-phosphate	(–)	(+)	(–)	(–)	(+)	(+)	(–)	(+)
DNA G + C content (mol%)	(64.1)	66.0–68.0	68.4–68.7	(68.9)	(65.6)	(66.1)	66.5	65.0–66.0

*Data from this study. Other data were taken from Yoon *et al.* (2006).

W1-2B^T, *Brevundimonas vesicularis* LMG 2350^T, *Brevundimonas intermedia* ATCC 15262^T, *Brevundimonas kwangchunensis* KSL-102^T and *Brevundimonas aurantiaca* DSM 4731^T, which exhibited 16S rRNA gene sequence similarities above 97 %. The extracted genomic DNAs were fragmented with *Hae*III for slot hybridization. The digested DNAs were serially diluted and loaded into slots (three replications for each sample) and their DNAs used individually as labelled DNA probes for cross-hybridization. Random-primed DNA labelling with digoxigenin-dUTP and hybridization (hybridization at 51.5 °C; washing at 68 °C) were performed using the DIG High Prime DNA labelling kit (Roche Applied Science) according to the instructions of the manufacturer and using standard procedures (Sambrook & Russell, 2001; Lim *et al.*, 2005). The signals of the series of dilutions were quantified using Bio-Rad GelDoc scanning software. The signals produced by self-hybridization were inferred as

100 % and the homology percentages were calculated from samples analysed in triplicate.

The neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequences indicated that strain EMB102^T formed a distinct phyletic lineage within the genus *Brevundimonas* (Fig. 2). The topologies of the phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that the isolate belongs to the genus *Brevundimonas* and can be differentiated from the other species of the genus (data not shown). Comparative 16S rRNA gene sequence analyses showed that the isolate was most closely related to *B. nasdae* W1-2B^T, *B. vesicularis* LMG 2350^T, *B. intermedia* ATCC 15262^T, *B. kwangchunensis* KSL-102^T and *B. aurantiaca* DSM 4731^T, with similarities of 97.5, 97.5, 97.3, 97.2 and 97.2 %, respectively. However, the values for

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain EMB102^T and related taxa. Bootstrap percentages (from 1000 replicates) are shown if greater than 50%. *Maricaulis maris* ATCC 15268^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

DNA–DNA relatedness between strain EMB102^T and the related *Brevundimonas* species were below 15.0%. Therefore, the physiological, biochemical and phylogenetic properties of strain EMB102^T support its description as a novel species within the genus *Brevundimonas*, for which the name *Brevundimonas aveniformis* sp. nov. is proposed.

Description of *Brevundimonas aveniformis* sp. nov.

Brevundimonas aveniformis (a.ven.i.for'mis. L. n. *avena* stalk; L. suff. *-formis* of the shape of; N.L. fem. adj. *aveniformis* stalk-shaped).

Cells are Gram-negative, rod-like bacteria that can produce stalks; the size of the main cell body is 0.3–0.4 × 1.0–2.0 µm. Colonies on R2A agar are white, slightly raised and circular with entire margins. Growth occurs optimally at 30 °C and pH 7.5–8.5. The optimum NaCl concentration is 0–1% (w/v); no growth occurs at ≥3%. Catalase- and

oxidase-positive. Nitrate is reduced to nitrite and nitrogen gas is produced. Hydrolyses casein, urea, tyrosine and gelatin, but not Tween 80 and 20, aesculin or starch. Negative for indole, H₂S and acetoin production and citrate utilization (API 20E). Produces acids from raffinose, myo-inositol, D-mannose and D-mannitol, but not from D-glucose, D-fructose, D-galactose, L-arabinose, melibiose, lactose, arbutin or salicin. Produces leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and urease, but not esterase (C4), lipase (C14), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Weak enzymic activities are observed for esterase lipase (C8), alkaline phosphatase, trypsin, tryptophan deaminase, α-chymotrypsin and acid phosphatase. The predominant polar lipid is phosphatidylglycerol. Utilizes glycogen, pyruvic acid methyl ester, β-hydroxybutyric acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic

acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-proline, L-serine, L-threonine and urocanic acid (Biolog GN2). Other organic substrates included in Biolog GN2 MicroPlate system are not utilized. The major isoprenoid quinone is Q-10. The major cellular fatty acids are C_{18:1ω7c}, C_{16:0} and C_{15:0}, and the main hydroxy fatty acid is iso-C_{17:0} 3-OH. The DNA G+C content is 64.1 mol% (HPLC).

The type strain, EMB102^T (=KCTC 12609^T=DSM 17977^T), was isolated from activated sludge that performed EBPR in a laboratory-scale sequencing batch reactor.

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