Shinella granuli gen. nov., sp. nov., and proposal of the reclassification of *Zoogloea ramigera* ATCC 19623 as *Shinella zoogloeoides* sp. nov.

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The taxonomic position of a novel bacterial strain, Ch06^T, isolated from an upflow anaerobic sludge blanket reactor was determined. Strain Ch06^T was Gram-negative, aerobic, motile and oxidase- and catalase-positive. A comparative 16S rRNA gene sequence analysis showed a clear affiliation of strain Ch06^T to the *Alphaproteobacteria* and it was most closely related to *Zoogloea ramigera* ATCC 19623 and *Mycoplana dimorpha* IAM 13154^T (97·9 and 96·3 % sequence similarity, respectively). The major respiratory quinone was Q-10 and the predominant fatty acids were $C_{16:0}$, 3-OH $C_{16:0}$, $C_{18:0}$, $C_{19:0}$ cyclo ω8c and summed feature 7 ($C_{18:1}\omega7c/\omega9t/\omega12t$, $C_{18:1}\omega7c/\omega9c/\omega12t$). On the basis of phenotypic, chemotaxonomic and phylogenetic characteristics, the novel isolate was assigned to a new genus, *Shinella* gen. nov., as *Shinella granuli* gen. nov., sp. nov. (type strain Ch06^T = KCTC 12237^T = JCM 13254^T). It is proposed that *Zoogloea ramigera* ATCC 19623 is reclassified into the novel genus *Shinella* as *Shinella zoogloeoides* sp. nov. (type strain ATCC 19623^T = IAM 12669^T = I-16-M^T).

In the course of a study on the culturable aerobic bacterial community in granules from an upflow anaerobic sludge blanket (UASB) reactor, a large number of novel bacterial strains were isolated (Bae *et al.*, 2005; La *et al.*, 2005). One of these isolates, designated Ch06^T, occupied a distinct phylogenetic lineage with the previously described strain *Zoogloea ramigera* ATCC 19623 (=IAM 12669) within the '*Rhizobiaceae* group' of the *Alphaproteobacteria* on the basis of 16S RNA gene sequence analysis.

Zoogloea ramigera, the type species of the genus *Zoogloea* (Unz, 1984), is defined as a Gram-negative, obligately aerobic, chemo-organotrophic, non-spore-forming, rod-shaped bacterium that produces a characteristic gelatinous matrix of finger-like projections, the so-called 'zoogloeal matrix' (Unz, 1984; Dugan *et al.*, 1992). Three strains of *Z. ramigera* are well-known through a number of experimental studies: the type strain ATCC 19544^T (=106^T; Unz, 1971), ATCC 19623 (=I-16-M; Crabtree & McCoy, 1967) and ATCC 25935 (=P. R. Dugan 115; Dugan *et al.*, 1992; Friedman & Dugan, 1968; Joyce & Dugan, 1970). The *Z. ramigera* type strain ATCC 19544^T and strain ATCC 19623 are known to be different (Rosselló-Mora *et al.*, 1993; Shin *et al.*, 1993) and the third strain has been reclassified as the type strain of *Duganella zoogloeoides* (Hiraishi *et al.*, 1997).

In this study, we report the results of a taxonomic examination of a newly isolated novel strain, Ch06^T, and of strain ATCC 19623.

For the isolation of aerobic bacteria, brownish-black granules (around 2 mm in diameter) from a brewery wastewater-treating UASB reactor, which had been operated anaerobically for 2 years, were homogenized by using an Ace homogenizer (Nihonseiki). The suspension was spread on R2A agar plates (Difco) after being serially diluted with 50 mM phosphate buffer (pH 7·0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto new plates and incubating them again under the same conditions. The purified colonies were tentatively identified by partial 16S rRNA gene sequences. Strain Ch06^T was one of the isolates that appeared dominantly on the plates under aerobic conditions. After primary isolation and purification on R2A agar plates (Difco), strains were cultivated at 30 °C on the same medium and stored at −70 °C in R2A broth supplemented with 20 % (v/v) glycerol.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim *et al.* (2005). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed using the

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Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ch06^T is AY995149.

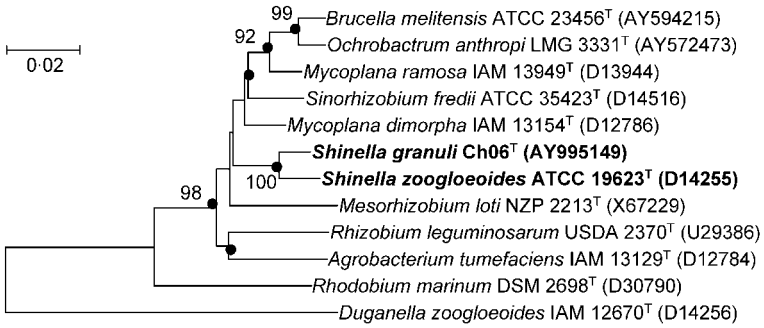

Fig. 1. Rooted phylogenetic tree based on the 16S rRNA gene sequences of strain Ch06^T and related bacteria in the *Alphaproteobacteria*. This tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with a Kimura (1983) two-parameter distance matrix and pairwise deletion. Dots indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 70 % are shown at the branch points. Bar, 0·02 substitutions per nucleotide position.

CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

Phylogenetic 16S rRNA gene sequence analyses indicated that strain Ch06^T is a member of the family *Rhizobiaceae* and forms a distinct cluster with strain ATCC 19623 (Fig. 1). Genomic DNA of strain Ch06^T was extracted and purified with the Qiagen genomic-tip system 100/G and was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The DNA G+C content was determined as described by Mesbah *et al.* (1989) using a reverse-phase HPLC. The DNA G+C content of strain Ch06^T was 66 mol%. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. The level of DNA–DNA relatedness between strains Ch06^T and ATCC 19623 was 33 %, suggesting that the strains are different at the species level (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The cellular fatty acids of strain Ch06^T, grown on trypticase soy agar (Difco) for 48 h, were saponified, methylated, extracted and identified by the Microbial Identification software package (Sasser, 1990). The major fatty acids of strain Ch06^T were $C_{16:0}$ (9·8–10·4 %), 3-OH $C_{16:0}$ (1·6–2·2 %), $C_{18:0}$ (1·6–2·6 %), $C_{19:0}$ cyclo ω8c (3·0–4·9 %) and summed feature 7 (75·8–76·9 %; $C_{18:1}\omega7c/\omega9t/\omega12t$, $C_{18:1}\omega7c/\omega9c/\omega12t$), showing a similar pattern to those of strain ATCC 19623, except for the presence of summed feature 3 ($C_{16:1}\omega7c$/15 : 0 iso 2-OH) (Table 1). Respiratory lipoquinones were analysed as described previously (Komagata & Suzuki, 1987); the major respiratory lipoquinone was ubiquinone-10 (Q-10).

Cell morphology and motility were observed under a Nikon light microscope (× 1000 magnification) with cells grown on R2A agar for 3 days at 28 °C. The presence of flagella was determined by transmission electron microscopy (JEM-10111; JEOL) after negative staining with uranyl acetate. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth was determined by monitoring the OD_{600}. Anaerobic growth was observed in serum bottles by adding thioglycolate (1 g l^{-1}) to R2A broth and substituting the upper air layer with nitrogen gas. Substrate utilization as the sole carbon source and some physiological characteristics were determined with API 32GN and API 20NE galleries according to the manufacturer's instructions (bioMérieux).

Table 1. Cellular fatty acid profiles of strain Ch06^T and type strains of related species

Species: 1, *Shinella granuli* sp. nov.; 2, *Shinella zoogloeoides* sp. nov.; 3, *Mycoplana dimorpha*; 4, *Sinorhizobium fredii* (data from Tighe *et al.*, 2000); 5, *Ochrobactrum anthropi* (Trujillo *et al.*, 2005). Fatty acids that account for <1·5 % of the total are not shown.

Fatty acid	1	2	3	4	5
16 : 0	9·8–10·4	13·4	6·2	5·12	7·2
16 : 0 3-OH	1·6–2·2	8·1	–	–	–
17 : 0	–	–	3·1	–	1·6
18 : 0	1·6–2·6	2·6	7·5	4·99	5·2
18 : 1 2-OH	–	–	–	–	2·4
19 : 0 cyclo ω8c	3·0–4·9	2·9	3·5	4·37	14·5
20 : 3ω6,9,12*c*	–	–	–	3·28	–
Summed feature 2*	–	–	–	–	1·6
Summed feature 3*	4·4–5·5	–	3·8	7·62	2·1
Summed feature 7*	75·8–76·9	72·9	76·1	71·45	62·8

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2: 12 : 0 unknown aldehyde with ECL 10.928/16 : 1 iso I/14 : 0 3-OH. Summed feature 3: 16 : 1ω7*c*/15 : 0 iso 2-OH. Summed feature 7: 18 : 1ω7*c*/ω9*t*/ω12*t*, 18 : 1ω7*c*/ω9*c*/ω12*t*.

Nitrate and nitrite reduction were confirmed by inoculating each strain into three 25 ml serum bottles containing 13 ml R2A medium. Nitrate and nitrite were added as KNO_3 and $NaNO_2$ at concentrations of 10 mM. The reduction of nitrate and nitrite was monitored by an ion chromatograph (790 personal IC; Metrohm) equipped with a conductivity detector and an anion exchange column (Metrosep Anion Supp 4; Metrohm). Nitrogen-fixing ability was determined by growth in 50 ml of a nitrogen-free medium (DSMZ medium no. 3) contained in a 500 ml Erlenmeyer flask. The primer system PolF–PolR (Poly *et al.*, 2001) was used to amplify a *nifH* gene as described by Im *et al.* (2004). DNA degradation [using DNA agar (Difco) supplemented with 0·01 % toluidine blue (Merck)], casein, cellulose and starch degradation (Atlas, 1993), lipid degradation (Kouker & Jaeger, 1987) and xylan degradation (Ten *et al.*, 2004) were also investigated; reactions were read after 5 days. Duplicate antibiotic sensitivity tests were performed using filter paper discs containing the following: streptomycin (5, 10 and 15 μg ml^{-1}), tetracycline (5, 10 and 15 μg^{-1}), kanamycin (1·0, 1·5 and 2·0 mg ml^{-1}) and ampicillin (20, 25 and 30 μg ml^{-1}) (Sigma). Discs were placed on R2A plates spread with Ch06^T culture and then incubated at 28 °C for 5 days.

Physiological, biochemical and morphological characteristics of the strains studied are listed under the species descriptions and are also given in Table 2. Phenotypic and chemotaxonomic examination shows that strains Ch06^T and ATCC 19623 share many common characteristics.

Table 2. Comparison of selected characteristics of *Shinella granuli* Ch06^T with those of its nearest phylogenetic neighbours within the '*Rhizobiaceae* group'

Species: 1, *Shinella granuli* sp. nov.; 2, *Shinella zoogloeoides* sp. nov.; 3, *Mycoplana dimorpha*; 4, *Sinorhizobium fredii* (data from de Lajudie *et al.*, 1994; Wei *et al.*, 2002; Willems *et al.*, 2003; Young *et al.*, 2001); 5, *Ochrobactrum anthropi* (de Lajudie *et al.*, 1994; Lebuhn *et al.*, 2000; Kämpfer *et al.*, 2003; Trujillo *et al.*, 2005). Data for taxa 1–3 are from this study. All strains were positive for assimilation of histidine, inositol, lactate, mannose, mannitol, proline and sorbitol and negative for assimilation of adipate, 3-hydroxybenzoate, suberate and phenylacetate and negative for gelatin hydrolysis. +, Positive; −, negative; W, weak.

Characteristic	1	2	3	4	5
Flagellation type	Polytrichous	Mono- and polytrichous	Peritrichous	Subpolar or peritrichous	Subpolar or peritrichous
Nitrate reduction	+	−	−	ND	+
Growth at:					
40 °C	+	−	−	−	ND
pH 10	+	+	W	+	ND
4 % NaCl	+	−	+	−	ND
β-D-Glucosidase	+	+	−	ND	−
β-Galactosidase	+	+	−	ND	−
Urease	+	−	−	−	+
Growth on:					
N-Acetylglucosamine	+	+	−	+	+
Alanine	+	−	+	−	+
Arabinose	+	+	−	+	+
Fucose	+	+	−	+	+
Gluconate	+	−	−	−	+
4-Hydroxybenzoate	+	−	−	ND	+
2-Ketogluconate	−	−	+	−	+
Malate	+	−	−	+	+
Maltose	+	+	−	+	+
Propionate	+	+	−	−	+
L-Rhamnose	+	+	−	+	+
D-Ribose	+	+	−	−	+
Salicin	+	−	−	−	−
Sucrose	+	+	−	+	+
Nitrogen fixation	−	−	−	+	ND
Mean DNA G+C content (mol%)	66	64	63–68	62	56–59

However, the strains differ with respect to nitrate reduction, growth at 40 °C, growth at 4 % NaCl, urease activity and their ability to assimilate carbon sources such as alanine, gluconate, 4-hydroxybenzoate, malate and salicin. Finally, the DNA–DNA relatedness value between the strains is low enough to differentiate them as separate species.

Phylogenetically, the two strains form a novel lineage of descent within the *Alphaproteobacteria* which is clustered with *Mycoplana dimorpha* IAM 13154^T (96·1–96·3 % similarity in 16S rRNA gene sequence) and *Sinorhizobium fredii* ATCC 35423^T (95·2–95·5 % similarity). The phylogenetic divergence and low level of similarity between 16S rRNA gene sequences support the affiliation of strains Ch06^T and ATCC 19623 into a novel genus. This is also supported by phenotypic and chemotaxonomic characteristics (Tables 1 and 2). The two strains can be differentiated from *Mycoplana dimorpha* IAM 13154^T by β-glucosidase and β-galactosidase activity and the ability to assimilate arabinose, fucose, 2-ketogluconate, maltose, *N*-acetylglucosamine, propionate, L-rhamnose, D-ribose and sucrose. The hydroxy fatty acid identified for the two strains was 3-OH $C_{16:0}$, which is a unique profile of the proposed new genus *Shinella* gen. nov., and different from those of other genera such as *Mycoplana*, *Sinorhizobium* and *Ochrobactrum* (Table 1). It is noted that, in some cases, this hydroxyl fatty acid is important for discriminating between genera, for example *Sinorhizobium* and *Rhizobium* (Tighe *et al.*, 2000). Moreover, the absence of cellular fatty acid $C_{17:0}$ also makes the genus *Shinella* different from the genera *Mycoplana* and *Ochrobactrum* (Table 1). Strains Ch06^T and ATCC 19623 can be differentiated from *Sinorhizobium fredii* ATCC 35423^T by nitrogen fixation and the ability to assimilate D-ribose and propionate. The DNA G+C content of strains Ch06^T and ATCC 19623 was 2–4 mol% higher than that of *Sinorhizobium* (Table 2). Moreover, strains Ch06^T and ATCC 19623 can be differentiated from *Ochrobactrum anthropi* LMG 3331^T by their relatively low DNA G+C content and by their phenotypic and chemotaxonomic features (Tables 1 and 2). On the basis of morphological, phylogenetic, chemotaxonomic and physiological data, we propose that strain Ch06^T is a member of a novel genus and species, *Shinella granuli* gen. nov., sp. nov. In addition, we propose the reclassification of *Zoogloea ramigera* ATCC 19623 to the genus *Shinella* as *Shinella zoogloeoides* sp. nov.

Description of *Shinella* gen. nov.

Shinella (Shi.nel′la. N.L. fem. dim. n. *Shinella* named after Yong-Kook Shin, for his contributions to reclassification of the genus *Zoogloea*).

Cells are Gram-negative, non-spore-forming, motile rods. Amorphous or finger-like flocculent growth occurs in liquid media. Catalase-, oxidase-, β-galactosidase- and β-glucosidase-positive. Predominant cellular fatty acids are $C_{16:0}$ and summed feature 7 ($C_{18:1}\omega7c/\omega9t/\omega12t$, $C_{18:1}\omega7c/\omega9c/\omega12t$) and $C_{16:0}$ 3-OH is the predominant hydroxy fatty acid. The main lipoquinone is Q-10. DNA G+C content is 64–66 mol%. 16S rRNA gene sequence analysis indicates that the genus *Shinella* is a member of the family *Rhizobiaceae* of the *Alphaproteobacteria*. The type species is *Shinella granuli*.

Description of *Shinella granuli* sp. nov.

Shinella granuli (gra.nu′li. L. gen. n. *granuli* of a small grain, pertaining to a granule, from which the type strain was isolated).

Characteristics are as given for the genus. In addition, cells are 0·2–0·5 μm in width and 4–6 μm in length. Motile by means of multiple polar flagella (Fig. 2). Colonies on R2A agar media are glistening, convex with an entire margin, viscous and pale-yellow. Growth occurs at 4–40 °C, with 1–4 % NaCl and at pH 6–10. Nitrogen fixation is negative. Does not hydrolyse starch, cellulose, xylan, protein, lipid, casein, gelatin or DNA. Does not utilize adipate, arginine, caparate, citrate, glycogen, 3-hydroxybenzoate, itaconate, 2-ketogluconate, 5-ketogluconate, malonate, melibiose, phenylacetate, suberate or valerate as sole carbon sources. Resistant to 50 μg ampicillin ml^{-1} and 15 μg tetracycline ml^{-1}, but sensitive to 5 μg streptomycin ml^{-1} and 1 mg kanamycin ml^{-1}. The predominant cellular fatty acids are $C_{16:0}$ (9·8–10·4 %), 3-OH $C_{16:0}$ (1·6–2·2 %), $C_{18:0}$ (1·6–2·6 %), $C_{19:0}$ cyclo $\omega8c$ (3·0–4·9 %) and summed feature 7 (75·8–76·9 %; $C_{18:1}\omega7c/\omega9t/\omega12t$, $C_{18:1}\omega7c/\omega9c/\omega12t$). The major respiratory quinone is Q-10. DNA G+C content is 66 mol%.

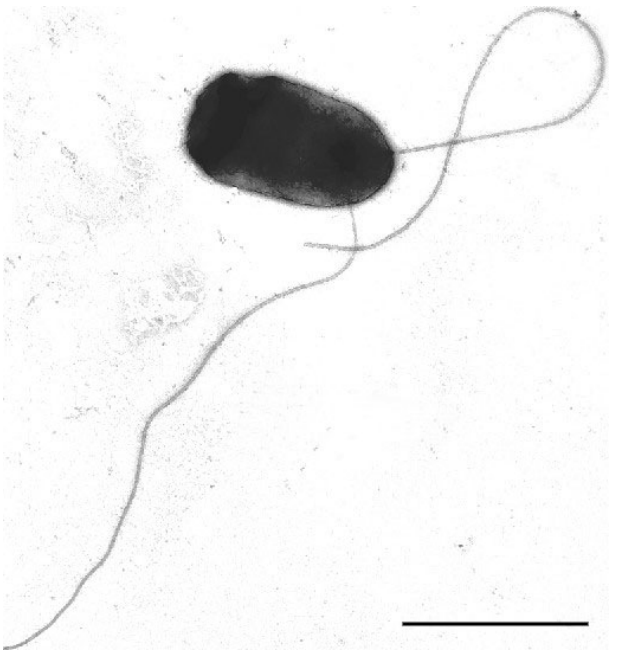

Fig. 2. Transmission electron micrograph of a motile cell of *Shinella granuli* Ch06^T. Bar, 1 μm.

The type strain, Ch06^T (=KCTC 12237^T=JCM 13254^T), was isolated from a UASB reactor.

Description of *Shinella zoogloeoides* sp. nov.

Shinella zoogloeoides (zoo.gloe.o′i.des. N.L. n. *Zoogloea* bacterial genus name; Gr. suff. *-oides* similar to; N.L. adj. *zoogloeoides* similar to *Zoogloea*).

The description is as given for the genus and by Shin *et al.* (1993) and Rosselló-Mora *et al.* (1993), with the addition that it is negative in tests for amylase, protease, lipase, cellulose, xylanase, DNase, gelatinase and urease. Nitrogen fixation is negative. Does not utilize adipate, alanine, arginine, caparate, citrate, glycogen, gluconate, 3-hydroxybenzoate, 4-hydroxybenzoate, itaconate, 2-ketogluconate, 5-ketogluconate, malate, malonate, melibiose, phenylacetate, salicin, suberate or valerate. The predominant cellular fatty acids are $C_{16:0}$ (13·4 %), 3-OH $C_{16:0}$ (8·1 %), $C_{18:0}$ (2·6 %), $C_{19:0}$ cyclo $\omega 8c$ (2·9 %) and summed feature 7 (72·9 %; $C_{18:1}\omega 7c$/$\omega 9t$/$\omega 12t$, $C_{18:1}\omega 7c$/$\omega 9c$/$\omega 12t$). The major respiratory quinone is Q-10. DNA G+C content is 64 mol%.

The type strain, ATCC 19623^T (=IAM 12669^T=I-16-M^T), was isolated from sewage treatment systems.

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