Kaistia granuli sp. nov., isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor

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A Gram-negative, chemo-organotrophic, non-motile, non-spore-forming, rod-shaped bacterium (designated strain Ko04^T) was isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor, and was investigated using a polyphasic taxonomic approach. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Ko04^T belongs to the order *Rhizobiales* in the *Alphaproteobacteria*. Comparative 16S rRNA gene sequence analysis showed that strain Ko04^T was most closely related to *Kaistia adipata* (97.5%) and that sequence similarities with other species of *Rhizobiales* with validly published names were less than 92.5%. The predominant ubiquinone was Q-10 and the major fatty acids were C_{18:1}ω7c/ω9t/ω12t, C_{19:0} cyclo ω8c and C_{18:0}. The G+C content of the genomic DNA of strain Ko04^T was 67.8 mol%. The level of DNA–DNA relatedness with *K. adipata* Chj404^T was 15%. The results of the genotypic analyses in combination with chemotaxonomic and physiological data demonstrated that strain Ko04^T represents a novel species within the genus *Kaistia*, for which the name *Kaistia granuli* sp. nov. is proposed. The type strain is Ko04^T (=KCTC 12575^T=LMG 23410^T).

The genus *Kaistia* was described recently by Im *et al.* (2004), and at present comprises only one species, *Kaistia adipata* (Im *et al.*, 2004). It was isolated from a sediment sample collected from an industrial stream. *K. adipata* was characterized as a Gram-negative, strictly aerobic, chemo-organotrophic, non-motile rod to coccus-shaped bacterium. Q-10 was the predominant quinone and C_{18:1}ω7c/ω9t/ω12t was the major fatty acid when *K. adipata* was grown on trypticase soy agar (TSA) for 24 h but, when grown on TSA for 48 h, C_{19:0} cyclo ω8c was the major fatty acid. According to the 16S rRNA gene sequence, the genus *Kaistia* belongs to the order *Rhizobiales* of the class *Alphaproteobacteria*.

During the course of studies on the culturable aerobic bacterial community in granules used to treat wastewater of brewing factories in Korea, a large number of novel bacterial strains were isolated (Im *et al.*, 2003). Some of these strains have already been classified as representing novel species (An *et al.*, 2006; Aslam *et al.*, 2005; Bae *et al.*, 2005; Kim *et al.*, 2005; La *et al.*, 2005). In this study, we have characterized one of these isolates, strain Ko04^T.

Phenotypic, chemotaxonomic and phylogenetic analyses established the affiliation of this isolate to the genus *Kaistia*. The data obtained in this study suggest that the isolate represents a novel species of this genus.

For the isolation of aerobic bacteria, brownish-black granules (about 2 mm in diameter) from an upflow anaerobic sludge blanket reactor used to treat brewery wastewater, which had been operated anaerobically for 2 years, were homogenized by using an Ace Homogenizer (Nihonseiki Co.). The suspension was spread on R2A agar plates (Scharlau) 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 being transferred onto new plates that were incubated again under the same conditions. The purified colonies were tentatively identified by partial sequences of the 16S rRNA gene. Strain Ko04^T was one of the isolates that appeared on the plates under aerobic conditions. The strain was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

Cell morphology and motility were observed under a Nikon light microscope (×1000 magnification) using cells

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ko04^T is AB244762.

grown for 2 days at 30 °C on R2A agar. The Gram reaction was determined by using the non-staining method as described by Buck (1982). Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined using 1% (w/v) tetramethyl-*p*-phenylenediamine. Growth at different temperatures (4, 15, 18, 25, 30, 37, 42 and 45 °C) and various pH values (pH 5.0–10.0 at intervals of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A agar supplemented with 1–10% (w/v) NaCl after 5 days incubation. Growth on nutrient agar, TSA and MacConkey agar was also evaluated, at 30 °C. Utilization of various substrates as sole carbon sources was investigated, together with some physiological characteristics, using API ID 32 GN, API 20NE and API ZYM galleries according to the instructions of the manufacturer (bioMérieux). Anaerobic growth was tested in serum bottles by addition of thioglycolate (1 g l⁻¹) to R2A broth and replacement of the upper air layer with nitrogen gas. Tests for the degradation of DNA [DNase agar (Scharlau), by flooding plates with 1 M HCl], casein, chitin, starch (Atlas 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were performed and evaluated after 10 days.

For phylogenetic analysis of strain Ko04^T, DNA was extracted using a genomic DNA extraction kit (Solgent) and PCR of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim *et al.* (2005). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank.

Multiple alignments were performed by using the program CLUSTAL_X (Thompson *et al.*, 1997). Gaps were edited in the program BioEdit (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) using the program MEGA 3 (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

The length of the almost-complete 16S rRNA gene sequence of strain Ko04^T was 1429 bp (bp 18–1512 with respect to the *Escherichia coli* numbering system). Sequence similarity calculations after neighbour-joining analysis indicated that the closest relative of strain Ko04^T was *K. adipata* Chj404^T (97.5%). Lower sequence similarities (92.5%) were found with other recognized species of *Rhizobiales*. The relationship between strain Ko04^T and members of the order *Rhizobiales* was also evident in the phylogenetic tree (Fig. 1). Strain Ko04^T and *K. adipata* Chj404^T formed a monophyletic clade supported by a high bootstrap value (100%), which was supported by the two types of tree-making methods employed.

For the measurement of the G+C content of the chromosomal DNA, genomic DNA of the novel strain was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides. The DNA G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v),

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain Ko04^T and related taxa. Solid circles at nodes indicate generic branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

evaporated under vacuum conditions and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane/quinone solution was purified using Sep-Pak Vac cartridges silica (Waters) and was subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). Cellular fatty acid profiles were determined for strain Ko04^T, grown under different conditions. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification system (MIDI). The fatty acids were analysed using a gas chromatograph (Hewlett Packard 6890) and were identified using the Microbial Identification Software package (Sasser, 1990).

The G+C content of the genomic DNA of strain Ko04^T was 67.8 mol%. Q-10 was the predominant respiratory ubiquinone. As shown in Table 1, the major fatty acids of strain Ko04^T grown on TSA for 24 h were summed feature 7 (C_{18:1}ω7c/ω9t/ω12t; 38.1%), C_{19:0} cyclo ω8c (35.9%) and C_{18:0} (15.7%). The profile of the whole-cell fatty acid composition changed with time. However, the total amount of C_{19:0} cyclo ω8c and C_{18:1}ω7c/ω9t/ω12t was similar. Thus we could infer that C_{18:1}ω7c/ω9t/ω12t was transformed to C_{19:0} cyclo ω8c when cells became older. Comparison of the fatty acid profiles of strain Ko04^T showed that they were similar to those of *K. adipata* Chj404^T analysed previously, although there were differences in the proportions of some fatty acids. DNA–DNA hybridization experiments were performed between strain Ko04^T and *K. adipata* Chj404^T using the method described by Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and micro-dilution wells.

Physiological, biochemical, and genotypic characteristics of strain Ko04^T are summarized in the species description,

Table 1. Cellular fatty acid profiles (%) of strain Ko04^T and *K. adipata* Chj404^T

Strains: 1, strain Ko04^T grown on TSA for 24 h; 2, strain Ko04^T grown on TSA for 48 h; 3, *K. adipata* Chj404^T grown on TSA for 24 h; 4, *K. adipata* Chj404^T grown on TSA for 48 h. Data for *K. adipata* are from Im *et al.* (2004). Major fatty acids are indicated in bold. Fatty acids that account for less than 0.5% of the total fatty acids are not shown.

Fatty acid	1	2	3	4
Saturated				
C _{16:0}	4.2	5.3	11.0	22.2
C _{17:0}	3.3	4.4	1.6	3.5
C _{18:0}	15.7	13.3	16.0	14.8
Unsaturated				
C _{18:1} ω9c			0.8	1.1
C _{20:1} ω9t	2.7			
C _{19:0} cyclo ω8c	35.9	69.4	19.7	42.2
Summed feature 7*	38.1	7.6	47.7	10.3

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 7 contains C_{18:1}ω7c and/or ω9t and/or ω12t.

and characteristics that differentiated it from *K. adipata* Chj404^T are presented in Table 2.

Strain Ko04^T showed 97.5% 16S rRNA gene similarity with respect to *K. adipata* Chj404^T. A similarity of 97% or greater is significant for possible species relatedness (Stackebrandt & Goebel, 1994) and in this case DNA–DNA hybridizations needed to be performed. A DNA–DNA relatedness of 70% is the generally accepted limit for species delineation (Wayne *et al.*, 1987). Strain Ko04^T exhibited 15% DNA–DNA relatedness with the type strain of *K. adipata*. This level is sufficiently low to permit the classification of strain Ko04^T as representing a distinct species (Wayne *et al.*, 1987).

Table 2. Comparison of selected characteristics of strain Ko04^T and *K. adipata* Chj404^T using API kits (API 20NE, API ID 32 GN, API 20E and API ZYM)

Data are from this study. Cells of both strains are Gram-negative, non-spore-forming, non-motile, rod-shaped and strict aerobes. Colonies of both strains are very similar: ivory-pigmented, round and raised with greasy surface. Both strains are positive for nitrate reduction to nitrite and for catalase, oxidase, urease, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase, and degradation of starch. Both strains are negative for degradation of xylan, cellulose, DNA and olive oil, and for indole production, H₂S production, acidification of glucose, and for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Both strains are positive for the assimilation of mannitol, D-glucose, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, rhamnose, N-acetyl-D-glucosamine, D-ribose, inositol, sucrose, maltose and D-mannose. Both strains are negative for assimilation of propionate, caprate, valerate, citrate, histidine, 4-hydroxybenzoate, itaconate, suberate, malonate, 3-hydroxybenzoate, L-serine, gluconate, caprate, adipate, malate and phenylacetate. +, Positive; –, negative; w, weakly positive or delayed response.

Characteristic	Strain Ko04 ^T	<i>K. adipata</i> Chj404 ^T
Growth at 37 °C	–	+
Utilization as carbon source		
2-Ketogluconate	w	+
3-Hydroxybutyrate	w	+
L-Proline	–	+
Acetate	+	–
L-Alanine	–	+
Glycogen	–	+
Enzyme activity		
Acid phosphatase	+	–
Naphthol-AS-BI-phosphohydrolase	+	–
DNA G + C content (mol%)	67.8	67.4*

*Data from Im *et al.* (2004).

On the basis of the morphological, physiological and chemotaxonomic characteristics, together with data from the 16S rRNA gene sequence comparisons described above, strain Ko04^T should be placed within a novel species, for which we propose the name *Kaistia granuli* sp. nov.

Description of *Kaistia granuli* sp. nov.

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

Cells are Gram-negative, chemo-organotrophic, strictly aerobic, non-spore-forming, rod-shaped and 0.4–0.6 µm in width and 0.9–1.5 µm in length when grown for 2 days at 30 °C on R2A agar. After 2 days incubation on R2A agar, colonies are ivory-pigmented, round and raised with a greasy surface, and 1.0–3.0 mm in diameter. Growth occurs at 18–30 °C, but not at 4 °C or above 37 °C. Growth occurs on TSA, R2A, nutrient agar and MacConkey agar. Grows at pH 5.0–8.0, with optimum growth at pH 6.5–7.0. Tolerates 2 % (w/v) NaCl. Substrate utilization, enzyme activity and other physiological characteristics are given in Table 2. Q-10 is the predominant ubiquinone, and C_{18:1}ω7c/ω9t/ω12t isomer, C_{19:0} cyclo ω8c and C_{18:0} are the major cellular fatty acids. The DNA G+C content of the type strain is 67.8 mol% (as determined by HPLC).

The type strain, Ko04^T (=KCTC 12575^T=LMG 23410^T), was isolated from granules used in a wastewater-treatment plant in Gongju, South Korea.

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