Chitinibacter alvei sp. nov., isolated from stream water

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A Gram-negative, aerobic bacterium, designated strain TNR-14^T, was isolated from water of a stream located in southern Taiwan. Cells were rod-shaped and motile by means of a polar flagellum. The isolate exhibited optimum growth at 20 °C, at pH 7.0 and in the absence of NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that the strain belonged to the genus *Chitinibacter* and was related most closely to the type strain of *Chitinibacter tainanensis* (96.0% similarity). The predominant cellular fatty acids were summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 54.03%) and C_{16:0} (27.22%). The DNA G+C content of strain TNR-14^T was 57.5 mol%. The results of physiological and biochemical tests and chemotaxonomic data allowed the clear phenotypic differentiation of the new isolate from *Chitinibacter tainanensis*. Strain TNR-14^T is therefore considered to represent a novel species of the genus *Chitinibacter*, for which the name *Chitinibacter alvei* sp. nov. is proposed. The type strain is TNR-14^T (=LMG 25206^T =DSM 22217^T =BCRC 17968^T).

The genus *Chitinibacter* belongs to the family *Neisseriaceae*, class *Betaproteobacteria*, and was proposed by Chern *et al.* (2004). At the time of writing, the genus comprises just one recognized species, *Chitinibacter tainanensis*, the type strain of which was isolated from soil and showed strong chitinolytic activity (Chern *et al.*, 2004). The aim of the present study was to determine the taxonomic position of a *Chitinibacter*-like isolate, strain TNR-14^T, isolated from a stream located in southern Taiwan.

A stream water sample was collected in a sterile bag (200 ml) from Maolin Township (Kaohsiung Prefecture, southern Taiwan), stored at 4 °C and transported to the laboratory within 2 h. The water sample was spread on R2A agar (BD Difco) plates by the standard dilution plating method. After incubation of the plates at 25 °C for 5 days, strain TNR-14^T was purified as single colonies. The strain was preserved at −80 °C as a 20% (v/v) glycerol suspension in agar-free R2A-based broth (R2B), which was prepared according to the manufacturer's formula (BD Difco), or by lyophilization with 20% (w/v) skimmed milk

as the cryopreservation solution. *C. tainanensis* S1^T was obtained from the Bioresource Collection and Research Centre, Taiwan (BCRC), and was used as a reference strain.

Strain TNR-14^T was examined for a broad range of phenotypic properties. Catalase, oxidase, DNase and lipase activity and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined according to standard methods (Gerhardt *et al.*, 1994). Hydrolysis of chitin was tested on agar plates that were supplemented with colloidal chitin (Chern *et al.*, 2004). Bacterial cells were observed by phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth to ascertain cell morphology. The motility of cells was tested by the hanging drop method. The Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. A Gram-stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction. Colony morphology was examined by using a stereoscopic microscope (SMZ 800; Nikon). The pH range for growth was examined in R2B prepared with the appropriate biological buffers (pH 4–10, at increments of 0.5 pH units) (Chung *et al.*, 1995). Tolerance to various

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TNR-14^T is FJ593907.

NaCl concentrations was tested in nutrient broth prepared according to the formula of the BD Difco medium, except that the NaCl concentration was altered as required (0, 0.5 and 1.0–10 %, w/v, at increments of 1.0 %). Growth at various temperatures (4–45 °C) was measured in R2B. Growth was determined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH, NaCl concentrations and temperatures. Anaerobic growth was checked on R2A agar by using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). The 16S rRNA gene sequence of strain TNR-14^T was obtained by using a DNA sequencer (ABI Prism 310; Applied Biosystems) and the sequence was then assembled by using the Fragment Assembly System program within the Wisconsin package, version 9.1 (GCG, 1995). An almost-complete 16S rRNA gene sequence (1405 nt) of strain TNR-14^T was compared against those available from the EzTaxon (Chun *et al.*, 2007), Ribosomal Database Project (Maidak *et al.*, 2001) and GenBank databases via the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignment, including strain TNR-14^T and its closest relatives, was performed by using BioEdit software (Hall, 1999) and MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm by using the Jukes & Cantor (1969) distance model; bootstrap analysis for the neighbour-joining tree was performed on the basis of 1000 resamplings. A comparison of the 16S rRNA gene sequence of strain TNR-14^T with those of members of genera of the family *Neisseriaceae* in the *Betaproteobacteria* showed that the strain fell within the evolutionary radiation occupied by the genus *Chitinibacter* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain TNR-14^T formed a coherent clade with *C. tainanensis* S1^T. A similar tree topology was obtained by using the maximum-parsimony

method. Based on pairwise 16S rRNA gene sequence comparisons, strain TNR-14^T was related most closely to *C. tainanensis* S1^T (96.0 % similarity). Levels of 16S rRNA gene sequence similarity between strain TNR-14^T and the type strains of other recognized species in the family *Neisseriaceae* were below 93.5 %.

The DNA G+C content of strain TNR-14^T was estimated as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by means of HPLC. The DNA G+C content of strain TNR-14^T was 57.5 ± 1.0 mol% (mean ± SD of 3 determinations), a value similar to that for *C. tainanensis* S1^T (56 mol%; Chern *et al.*, 2004).

Biomass of strain TNR-14^T and of *C. tainanensis* S1^T for fatty acid analyses was harvested after growth on R2A agar at 20 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI) (Sasser, 1990). The predominant fatty acid constituents of strain TNR-14^T were summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 54.03 % of the total) and C_{16:0} (27.22 %) (Table 1). The fatty acid composition of *C. tainanensis* in this study differed slightly from the original description of *C. tainanensis* S1^T (Chern *et al.*, 2004), which can be attributed to the variation in culture conditions (Luria–Bertani, 30 °C, 48 h).

Isoprenoid quinones were purified by the methods outlined by Minnikin *et al.* (1984) and were analysed by HPLC as described by Collins (1985). Ubiquinone Q-8 was the major quinone (92 % of the total quinones).

DNA–DNA hybridization experiments were performed with strain TNR-14^T and *C. tainanensis* S1^T by using the method described by Ezaki *et al.* (1989). The level of DNA–DNA relatedness between strain TNR-14^T and *C. tainanensis* S1^T was 17.6 ± 10.5 % (mean ± SD of 3 determinations; reciprocal, 24.5 ± 6.8 %), clearly below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences and reconstructed by using the neighbour-joining algorithm with the distance model of Jukes & Cantor (1969), showing the position of strain TNR-14^T within the genus *Chitinibacter*. GenBank accession numbers are shown in parentheses. Bootstrap percentages (based on 1000 resampled data-sets) only above 50 % are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

Table 1. Major fatty acids of strain TNR-14^T and *Chitinibacter tainanensis* S1^T

Values are the percentages of the total fatty acids. The two strains were grown on R2A agar at 20 °C for 3 days. Values in parentheses are from Chern *et al.* (2004) for *C. tainanensis* S1^T (Luria–Bertani, 30 °C, 48 h).

Fatty acid	TNR-14 ^T	<i>C. tainanensis</i> S1 ^T
C _{12:0}	3.29	3.05 (2.45)
C _{12:0} 3-OH	4.06	5.20 (3.87)
C _{14:0}	2.46	1.32 (0.46)
C _{16:1} ω5c	0.46	0.54 (0.62)
C _{16:0}	27.22	22.99 (16.72)
C _{16:0} 3-OH	1.87	2.20 (1.33)
C _{18:0}	1.51	3.78 (0.79)
Summed feature 3*	54.03	50.14 (48.45)
Summed feature 8*	3.17	7.70 (19.85)

*Summed feature 3 comprised C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 8 comprised C_{18:1}ω7c and/or C_{18:1}ω6c.

The commercially available API 20NE (bioMérieux), API ZYM (bioMérieux) and MicroPlate GN2 (Biolog) micro-test systems were used to determine the biochemical properties, enzyme activities and carbohydrate utilization pattern of strain TNR-14^T. Tests were carried out according to the methods outlined by the manufacturers. Sensitivity to antibiotics was examined after spreading cells (0.5 McFarland standard) on R2A agar and placing antibiotic discs containing the following compounds: ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 µg), rifampicin (5 µg), streptomycin (10 µg), sulphamethoxazole (25 µg) and tetracycline (30 µg). The effects of the antibiotics on cell growth were assessed after 3 days of incubation, and susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. Cells were scored as susceptible if the distance was greater than 3 mm, as moderately susceptible if the distance was 1–3 mm and as resistant if the distance was less than 1 mm. Detailed results from the phenotypic and biochemical analyses of strain TNR-14^T are provided in Table 2 and in the species description below. Phenotypic characteristics that serve to differentiate strain TNR-14^T from the type strain of *C. tainanensis* are presented in Table 2.

On the basis of 16S rRNA gene sequence comparisons, strain TNR-14^T occupies a distinct position within the genus *Chitinibacter*. This genotypic insight was supported by the unique combination of chemotaxonomic characteristics (Tables 1 and 2) and biochemical traits (Table 2). It is thus clear from the genotypic and phenotypic data presented that strain TNR-14^T represents a novel species of the genus *Chitinibacter*, for which we propose the name *Chitinibacter alvei* sp. nov.

Table 2. Differential characteristics between strain TNR-14^T and *Chitinibacter tainanensis* S1^T

Data, except for DNA G+C content of *C. tainanensis* (Chern *et al.*, 2004), are from the present study. +, Positive reaction or growth; –, negative reaction or no growth; w, weakly positive reaction.

Characteristic	TNR-14 ^T	<i>C. tainanensis</i> S1 ^T
Growth at:		
37 °C	–	+
pH 9	–	+
Hydrolysis of:		
Chitin	w	+
Casein	–	+
DNA	+	–
Tween 20	+	–
Tween 40	+	–
Tween 60	+	–
API ZYM results		
Alkaline phosphatase	+	–
Esterase lipase (C8)	+	–
Valine arylamidase	–	+
Cystine arylamidase	–	+
Acid phosphatase	–	+
Naphthol-AS-BI-phosphohydrolase	–	+
α-Glucosidase	+	–
API 20NE results		
Nitrate reduction	+	–
β-Glucosidase	w	–
Protease	+	–
Oxidation of (Biolog GN2):		
Dextrin	w	–
Glycogen	w	–
Tween 40	+	–
Tween 80	w	–
Maltose	+	–
Pyruvic acid methyl ester	w	–
Citric acid	+	–
Malonic acid	–	w
Succinic acid	+	–
L-Alaninamide	+	–
L-Alanine	+	–
L-Alanyl glycine	+	–
L-Asparagine	+	–
L-Aspartic acid	+	–
L-Glutamic acid	w	–
Glycyl L-aspartic acid	+	–
Glycyl L-glutamic acid	+	–
L-Histidine	+	–
L-Phenylalanine	w	–
L-Serine	+	–
Glycerol	+	–
DNA G+C content (mol%)	57.5	56

Description of *Chitinibacter alvei* sp. nov.

Chitinibacter alvei (al've.i. L. gen. n. *alvei* bed of a river, referring to the location from where the type strain was isolated.)

Cells are Gram-negative rods (0.5–0.7 µm in width and 0.8–2.5 µm in length) that are highly motile by means of a single polar flagellum. Colonies on R2A agar are milky white, circular and slightly convex with entire edges. Colonies are approximately 1.0–2.0 mm in diameter after growth on R2A agar for 72 h at 20 °C. Growth occurs at 10–35 °C, at pH 6–8 and in the presence of 0–1 % NaCl. Optimum growth occurs at 20 °C, at pH 7.0 and in the absence of NaCl. Cells are strictly aerobic. Positive for oxidase, catalase, DNase, and hydrolysis of chitin and Tweens 20, 40 and 60. Negative for lipase (corn oil) and hydrolysis of casein, starch and Tween 80. Positive for nitrate reduction, aesculin hydrolysis, gelatin hydrolysis, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, α -glucosidase and *N*-acetyl- β -glucosaminidase. Negative for indole production, glucose fermentation, arginine dihydrolase, urease, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase. Utilizes the following carbon substrates (Biolog GN2): dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, D-fructose, α -D-glucose, maltose, D-mannose, pyruvic acid methyl ester, citric acid, D-gluconic acid, succinic 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-phenylalanine, D-serine, L-serine and glycerol. Unable to utilize α -cyclodextrin, *N*-acetyl-D-galactosamine, adonitol, D-arabitol, cellobiose, i-erythritol, L-fucose, D-galactose, gentiobiose, *myo*-inositol, lactose, lactulose, D-mannitol, melibiose, methyl β -D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, succinic acid mono-methyl ester, acetic acid, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-hydroxyproline, L-leucine, L-ornithine, L-proline, L-pyrroglutamic acid, D-serine, DL-carnitine, γ -amino-butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate or D-glucose 6-phosphate. Susceptible to chloramphenicol, kanamycin, nalidixic acid, novobiocin, rifampicin, sulphamethoxazole and tetracycline. Moderately susceptible to streptomycin. Resistant to ampicillin, gentamicin and penicillin G. The predominant fatty acids (>5 %) are summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c) and C_{16:0}. The DNA G + C content of the type strain is 57.5 mol%.

The type strain, TNR-14^T (=LMG 25206^T =DSM 22217^T =BCRC 17968^T), was isolated from a stream located in southern Taiwan.

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