Belliella pelovolcani sp. nov., isolated from a mud-volcano in Taiwan

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Strain CC-SAL-25^T, a non-motile, Gram-negative, red-pigmented bacterium, was isolated from a rare mud-volcano, located in Wandan, Pingtung County, Taiwan. Studies based on 16S rRNA gene sequences showed that the strain clustered closely with *Belliella baltica* BA143^T (95.4 % sequence similarity). A menaquinone with seven isoprene units (MK-7) was the major respiratory quinone. The fatty acid profile was slightly different from that of *B. baltica* BA143^T. The results of the physiological and biochemical tests showed that strain CC-SAL-25^T could be clearly differentiated from recognized *Belliella* species based on phenotypic properties. It was evident from the genotypic and phenotypic data that strain CC-SAL-25^T should be classified as representing a second novel species in the genus *Belliella*. The name proposed for this taxon is *Belliella pelovolcani* sp. nov., and the type strain is CC-SAL-25^T (=BCRC 17883^T=KCTC 13248^T).

During investigations on the biodiversity of bacteria associated with a rare mud-volcano (mud basin) located in Liyushan (Mt. Carp), Wandan Township, Pingtung County, Taiwan [22° 56' N 120° 46' E; temperature, 38 °C; pH 8.2; alkalinity, 3.3 mM; Cl 13 mM; SO₄ 1020 µM; Na 10 mM (You *et al.*, 2004)], strain CC-SAL-25^T was isolated and maintained on marine agar 2216 (MA; Difco) after incubation at 37 °C for 48 h. Subcultivation was done on MA at 37 °C for 2–3 days. On this medium, strain CC-SAL-25^T was able to grow at 25–40 °C, but not at 45 or 20 °C. The organism was able to grow on nutrient agar (NA; HI-MEDIA), R2A agar (Difco) and MacConkey agar (bioMérieux). Strain CC-SAL-25^T was preserved at –80 °C in nutrient broth [marine broth (MB); Difco] with 20 % (v/v) glycerol or by lyophilization.

Gram-staining was performed as described by Gerhardt *et al.* (1994). The presence of poly-β-hydroxybutyrate granule accumulation was assessed using light microscopy (Zeiss model A3000; ×1000; Taiwan Instrument Co.) after the cells were stained with Sudan black. Phenotypic characteristics, biochemical tests, carbon source utilization (Biolog-GN), API ZYM enzyme profiles (bioMérieux), API 20E (bioMérieux), API 20NE (bioMérieux), API 50 CH (bioMérieux) and ATB-PSE (bioMérieux) were investi-

gated. For the guanine-plus-cytosine (G+C) content calculations, a DNA sample was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using high-performance liquid chromatography (HPLC). The G+C content of the DNA of strain CC-SAL-25^T was 40 ± 1 mol% (mean ± SD).

Cell morphology was observed by using light microscopy (Zeiss model A3000; ×1000), with cells that had been grown for 48 h at 37 °C on MA. The results are given in the species description. The pH range for growth was determined by measuring the optical density (wavelength, 595 nm) of a culture grown in MB that was adjusted prior to sterilization to various pH values (pH 3–11, at intervals of 0.5 pH units) using appropriate biological buffers (Chung *et al.*, 1995). Verification of the pH values after autoclaving revealed only minor changes. Growth at various temperatures (10–55 °C) was measured in MB. Anaerobic growth was checked by using the Oxoid AnaeroGen system (Miller *et al.*, 1995) in MB. Growth was recorded by measuring the optical density (OD₅₉₅) of the culture with respect to time. Cellular pigments were extracted with acetone/methanol (7:2, v/v), from cultures grown on MA and the absorption spectra were determined with a scanning UV/visible spectrophotometer (Hitachi; Japan). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992) and Bernardet *et al.* (2002).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-SAL-25^T is EU685336.

The 16S rRNA gene sequence of strain CC-SAL-25^T was determined and analysed as described previously (Young *et al.*, 2005). Analysis of the sequence data was performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar *et al.*, 2001), after multiple alignments of data using CLUSTAL_X (Thompson *et al.*, 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining (Fig. 1), and a discrete-character-based maximum-parsimony method were used. In each case bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain CC-SAL-25^T obtained was a continuous stretch of 1444 bp. Sequence similarity calculations indicated that strain CC-SAL-25^T showed greatest similarity to the species *Belliella baltica* (95.4%; GenBank accession no. AJ564643), which to-date is the only recognized species in the genus *Belliella* (Brettar *et al.*, 2004) in the *Cytophaga-Flavobacterium-Bacteroides* group, whereas lower sequence similarities

(<94.5%) were found with all other species shown in Fig. 1.

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The fatty acid profile of strain CC-SAL-25^T was consistent with that reported for *B. baltica* BA134^T (Brettar *et al.*, 2004), but the amounts of the fatty acids iso-C_{15:1} G, iso-C_{15:0} and C_{15:1}ω6c differed (Table 1).

Respiratory quinones of strain CC-SAL-25^T were extracted, separated and identified as described by Minnikin *et al.* (1984) and analysed by HPLC as described by Collins & Jones (1980). A menaquinone with seven isoprene units (MK-7) was the predominant respiratory quinone.

DNA–DNA relatedness experiments were not carried out between strain CC-SAL-25^T and its closest phylogenetic neighbour *B. baltica* BA134^T as the level of 16S rRNA gene

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the GenBank/EMBL/DBJ databases (accession numbers are given in parentheses) constructed after multiple alignments of data by using CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) and clustering with the neighbour-joining method were performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar *et al.*, 2001). Percentage bootstrap values based on 1000 replications are given at branch points. Bar, 0.02 nucleotide substitutions per nucleotide position.

Table 1. Fatty acid compositions of strain CC-SAL-25^T and *B. baltica* BA134^T

Strains: 1, CC-SAL-25^T (*Belliella pelovolcani* sp. nov.); 2, *B. baltica* BA134^T (data in parentheses are from Brettar *et al.*, 2004). Both strains were grown on MA at 30 °C for 48 h (this study). Values are percentages of the total fatty acid content. Fatty acids of less than 0.5 % are not shown. Values for predominant fatty acids are shown in bold. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. *cis* isomer is indicated by the suffix *c*. ND, Not determined.

Fatty acid	1	2
Saturated (straight-chain)		
C _{13:1} (at 12–13)	0.9	0.5 (ND)
C _{15:0}	0.5	0.7 (3.8)
C _{16:0}	1.5	1.2 (ND)
Saturated (branched)		
iso-C _{17:0}	0.6	0.7 (0.5)
iso-C _{14:0}	1.3	0.7 (1.8)
iso-C_{15:1} G	1.9	9.9 (10.1)
iso-C_{15:0}	31.2	23.9 (18.9)
iso-C _{16:0}	2.2	1.9 (2.8)
iso-C _{16:1} H	1.7	2.9 (3.2)
anteiso-C _{15:0}	2.9	5.4 (4.8)
Summed feature 4*	ND	4.4 (3.9)
Unsaturated		
C _{15:1} ω 6c	6.4	1.2 (2.5)
C _{16:1} ω 5c	4.2	4.9 (2)
C_{17:1}ω6c	7.9	5.6 (9.8)
C _{17:1} ω 8c	1.1	1 (1.5)
iso-C_{17:1}ω9c	4.4	7.3 (10.1)
Hydroxy		
C _{16:0} 3-OH	2.5	1.9 (ND)
iso-C _{15:0} 2-OH	1.2	0.9 (0.9)
iso-C _{15:0} 3-OH	4.2	3.9 (2.3)
iso-C _{16:0} 3-OH	1.1	0.7 (ND)
iso-C_{17:0} 3-OH	6.7	5.2 (3.0)
Summed feature 3*	12.3	12.7 (7)

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains one or more of the fatty acids C_{16:1} ω 7c and iso-C_{15:0} 2-OH. Summed feature 4 contains iso-C_{17:1} B/iso I.

sequence similarity between the strains was less than 96 %, which is below the cutoff value (97 %) suggested by Stackebrandt & Goebel (1994) for genomic distinction of species.

The results of the physiological characterization are given in the species description and in Table 2. Strain CC-SAL-25^T was capable of producing acid from various carbohydrates, but carbon substrate oxidation tests with organic acids as substrates showed few positive results.

Strain CC-SAL-25^T could be differentiated from *B. baltica* BA134^T, according to both phenotype (differential biochemical tests are given in Table 2) and genotype, and

Table 2. Genotypic, phenotypic and nutritional characteristics that distinguish strain CC-SAL-25^T (*Belliella pelovolcani* sp. nov.) from *B. baltica* BA134^T

Strains: 1, CC-SAL-25^T (*Belliella pelovolcani* sp. nov.); 2, *B. baltica* BA134^T (data from Brettar *et al.*, 2004). +, Positive; –, negative; w, weakly positive.

Characteristic	1	2
Cell size (μ m)	0.3 $\times$ 2.0–5.0	0.3–0.7 $\times$ 1.5–3.0
Colony colour	Pink/red	Pink/orange
Temperature range for growth (°C)	25–40	4–37
pH range for growth	6–9	6–10
DNA G + C content (mol%)	40 $\pm$ 1	35.3
API 50 CH		
D-Fructose	–	w
D-Galactose	–	+
D-Glucose	–	+
Methyl	+	–
α -D-mannopyranoside		
Methyl α -D-glucopyranoside	+	–
N-Acetylglucosamine	+	–
Amygdalin	+	–
Arbutin	+	–
Salicin	+	w
Inulin	+	–
Raffinose	+	w
Glycogen	+	w
Xylitol	+	–
Gentiobiose	+	–
Turanose	+	–
D-Lyxose	+	–
D-Tagatose	+	–
D-Fucose	+	–
L-Fucose	+	–
D-Arabitol	+	–
Potassium gluconate	+	–
Potassium 2-ketogluconate	+	–
Potassium 5-ketogluconate	+	–
API 20NE		
Nitrate reduction to nitrite	–	+
API ZYM		
Esterase lipase	+	w

represents a second member of the genus *Belliella*, for which we propose the name *Belliella pelovolcani* sp. nov.

Description of *Belliella pelovolcani* sp. nov.

Belliella pelovolcani (pe.lo.vol.ca'ni. Gr. n. *pelos* mud; L. n. *Volcanus* god of fire, volcano; N.L. gen. n. *pelovolcani* of a mud-volcano, from which the type strain was isolated).

Cells are Gram-negative, aerobic, rod-shaped, non-motile and non-spore-forming, 2.0–5.0 μ m in length and 0.3 μ m in diameter. Good growth occurs after 48 h incubation on MA at 37 °C. Colonies on marine agar at 37 °C are reddish/pink-pigmented, smooth, shiny and convex with spreading

edges, 1.0–2.0 mm in diameter and are non-fluorescent. Cellular pigments (red) present are carotenoids but not flexirubin. Optimal temperature for growth is 37 °C. Optimal pH for growth is 8.0; growth occurs at pH 6.0–9.0 but not at pH 5.0 or 10. Growth occurs in the presence of 0.5–6 % (w/v) NaCl; optimal growth occurs in the presence of 3 % (w/v) NaCl. Does not grow anaerobically on MA supplemented with nitrate. Poly- β -hydroxybutyrate granules are not accumulated. Oxidase- and catalase-positive. Does not reduce nitrate to nitrite. The following carbon sources are oxidized (positive with the Biolog GN2 system): α -cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, L-arabinose, cellobiose, i-erythritol, D-fructose, D-galactose, gentiobiose, α -D-glucose, α -D-lactose, lactulose, maltose, D-mannose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, sucrose, trehalose, turanose, succinic acid monomethyl ester, acetic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, L-ornithine, L-threonine, inosine, uridine, thymidine, α -D-glucose 1-phosphate and D-glucose 6-phosphate. The following substrates are not oxidized as carbon sources (negative with the Biolog GP2 system): *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, L-fucose, *myo*-inositol, D-mannitol, L-rhamnose, D-sorbitol, xylitol, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-gluconic acid, 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, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-proline, L-pyroglyutamic acid, D-serine, L-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and DL- α -glycerol phosphate. In API 20E (bioMérieux), positive for β -galactosidase, H₂S production, citrate utilization, acetoin production and gelatinase. In API 20NE, positive for aesculin, gelatinase and β -galactosidase. In API ZYM, positive for alkaline and acid phosphatase, butyrate esterase, caprylate esterase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-B-1-phosphohydrolase, β -galactosidase and α -glucosidase. Sensitive to the following antibiotics: ampicillin + sulbactam, ticarcillin, ticarcillin-clavulonic acid, piperacillin, piperacillin + tazobactam, cefepime, imipenem, meropenem, cotrimoxazole, ciprofloxacin; resistant to ceftazidime, amikacin, gentamicin, tobramycin and colistin (ATB-PSE 5). Additional phenotypic features are given in Table 2. The detailed fatty acid profile is given in Table 1.

The type strain, CC-SAL-25^T (=BCRC 17883^T=KCTC 13248^T), was isolated from a mud-volcano in Taiwan.

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