Cloacibacterium rupense sp. nov., isolated from freshwater lake sediment

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A Gram-negative, yellow-pigmented bacterium, designated strain R2A-16^T, was isolated from sediment of Rupa Lake in Nepal and analysed using a polyphasic taxonomic approach. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain R2A-16^T is affiliated to the genus *Cloacibacterium* of the family *Flavobacteriaceae*; 16S rRNA gene sequence similarity between strain R2A-16^T and *Cloacibacterium normanense* CCUG 46293^T was 98.07 %. The isolate contained iso-C_{15:0} (35.6 %) as the major fatty acid and menaquinone MK-6 as the predominant respiratory quinone. The G + C content of the genomic DNA was 33.3 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain R2A-16^T represents a novel species of the genus *Cloacibacterium*, for which the name *Cloacibacterium rupense* sp. nov. is proposed; the type strain is R2A-16^T (=CGMCC 1.7656^T =NBRC 104931^T).

The genus *Cloacibacterium* of the family *Flavobacteriaceae* was created by Allen *et al.* (2006) and currently comprises a single species, *Cloacibacterium normanense*. During studies of the bacterial diversity of sediment of Rupa Lake located in southern Nepal, a novel bacterium, strain R2A-16^T, was isolated. Strain R2A-16^T was considered to be a *Cloacibacterium*-like strain based on the results of the 16S rRNA gene sequence analysis. The aim of this study was to describe the exact taxonomic position of strain R2A-16^T using a polyphasic taxonomic approach.

Strain R2A-16^T was isolated on R2A agar incubated at 37 °C for 48 h. The recommended media and growth conditions were used to culture *C. normanense* DSM 15886^T (Allen *et al.*, 2006), used as a reference strain. Cell morphology of both strains was determined using cells grown on R2A medium. For fatty acid methyl ester analysis, strain R2A-16^T was cultivated on tryptic soy agar (Difco) at 37 °C for 24 h for direct comparison with reference strains. Cell biomass for quinone analysis and for DNA extraction was obtained from cultures in liquid R2A medium. The Gram reaction was assessed as described by Gerhardt *et al.* (1994) and by the KOH lysis method (Gregersen, 1978). Flexirubin-type pigments were detected according to the method of Fautz & Reichenbach (1980). Further pigment analysis was performed as described by

Allen *et al.* (2006). Catalase activity was determined by means of bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined from the oxidation of 1 % *p*-aminodimethylaniline oxalate. Hydrolysis of starch was tested on starch agar (Difco). Nitrate reduction and hydrolysis of casein, aesculin, urea, DNA and gelatin were examined according to Dong & Cai (2001). The pH range and optimum was determined according to the method of Allen *et al.* (2006). Tests for carbohydrate utilization were performed in a liquid medium containing 2.0 g (NH₄)₂SO₄, 0.5 g NaH₂PO₄, 0.5 g K₂HPO₄, 0.2 g MgSO₄, 0.02 % (w/v) yeast extract and 0.5 % (w/v) carbohydrate dissolved in 1 l distilled water. Media were incubated at <37 °C and 30 °C for strains R2A-16^T and NRS1^T, respectively, and results were determined spectrophotometrically by testing OD₆₆₀ after 3 days. Other tests, including anaerobic growth, antibiotic sensitivity and characteristics listed in Table 1 and the species description, were performed according to Dong & Cai (2001); *C. normanense* DSM 15886^T was also tested as a control.

For quantitative analysis of cellular fatty acid composition, fatty acid methyl ester mixtures were prepared and identified following the manufacturer's instructions for the Microbial Identification System (MIDI, 2002) and identified by using the Microbial Identification software package (Sasser, 1990). For analysis of isoprenoid quinones, cells grown aerobically were freeze-dried and the lipid fraction was extracted according to Collins (1985) and analysed by HPLC as described by Groth *et al.* (1997). Genomic DNA was prepared according to the method of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R2A-16^T is EU581834.

Maximum-parsimony and minimum-evolution 16S rRNA gene sequence-based phylogenetic trees are available as supplementary material with the online version of this paper.

Table 1. Differential characteristics of strain R2A-16^T and *C. normanense* DSM 15886^T

Data for *C. normanense* DSM 15886^T match those reported by Allen *et al.* (2006) unless indicated.

Characteristic	R2A-16 ^T	<i>C. normanense</i> DSM 15886 ^T
Colony pigmentation	Yellow	Light yellow*
Motility	+	—
Anaerobic growth	—	+
Optimal growth temperature (°C)	37	30
Hydrolysis of:		
DNA	—	+
Casein	—	+
Production of:		
Indole	—	+
Urease	+	—
Sensitivity to:		
Carbenicillin	—	+
Tetracycline	—	+
DNA G + C content (mol%)	33.3	31.0
Isolation source	Lake sediment	Municipal wastewater

*Reported as yellow (colony pigmentation) and weak (DNA hydrolysis) by Allen *et al.* (2006).

Marmur (1961). The G+C content was analysed by the thermal denaturation method using a BIO-20 UV spectrophotometer according to De Ley *et al.* (1970). For the calculation of DNA G+C content, the equation of De Ley *et al.* (1970) was used and corrected using genomic DNA of *Escherichia coli* K-12 as a reference. DNA–DNA hybridization experiments were carried out spectrophotometrically (De Ley *et al.*, 1970).

The 16S rRNA gene fragment of strain R2A-16^T was amplified using individual bacterial colony PCR (Güssow & Clackson, 1989). The primers used for PCR were 27F (5'-GAGAGTTTGATCCTGGCTCAG) and 1495R (5'-CT-ACGGCTACCTTGTTACGA) (*E. coli* numbering; Brosius *et al.*, 1978). PCR products were sequenced using DNA Sequencer 3730 with the software provided by the manufacturer (Applied Biosystems). An almost-complete 16S rRNA gene sequence was compared initially with reference sequences in GenBank using BLAST (Altschul *et al.*, 1997).

Sequence alignments were carried out using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Similarities (%) were calculated by the method of Jukes & Cantor (1969) in the program CLUSTAL W. Phylogenetic analysis of multiple sequence alignments was performed with MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic tree construction was carried out by using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Kluge & Farris, 1969) methods.

Details of the cultural, physiological and biochemical characteristics of strain R2A-16^T are given in the species description and in Table 1. The cellular fatty acid profile of strain R2A-16^T was characterized by the predominance of iso-C_{15:0} (35.6 %) and was similar to that of *C. normanense* NRS1^T. However, R2A-16^T differed from *C. normanense* NRS1^T in containing C_{14:0}, C_{15:0} 2-OH, C_{16:0}, C_{18:0} and summed feature 3 and lacking C_{12:0} 3-OH, C_{16:0} 3-OH and C_{16:1ω5c} (Table 2). The predominant respiratory quinone was menaquinone MK-6. The DNA G+C content was 33.3 mol%. DNA–DNA hybridization between the novel isolate and *C. normanense* DSM 15886^T was 17 %.

The almost-complete 16S rRNA gene sequence (1399 bp) of strain R2A-16^T was determined. 16S rRNA gene sequence similarity between R2A-16^T and *C. normanense* CCUG 46293^T was 98.07 %. Sequence similarities with all other species of the family *Flavobacteriaceae* included in the phylogenetic analysis were below 94.7 %. In the phylogenetic tree based on the neighbour-joining algorithm, strain R2A-16^T clustered with *C. normanense* CCUG 46293^T (Fig. 1). Similar tree topologies were also found in trees generated with the maximum-parsimony and minimum-evolution

Table 2. Cellular fatty acid composition of strain R2A-16^T and *C. normanense* NRS1^T

Values are percentages of total fatty acids. —, Not detected or less than 1 %. Data for *C. normanense* NRS1^T were taken from Allen *et al.* (2006).

Fatty acid	R2A-16 ^T	<i>C. normanense</i> NRS1 ^T
C _{12:0} 3-OH	—	2.1
iso-C _{13:0}	3.0	7.9
anteiso-C _{13:0}	—	1.1
iso-C _{14:0}	1.8	3.3
C _{14:0}	1.5	—
C _{15:0}	1.7	1.1
iso-C _{15:0}	35.6	40.8
anteiso-C _{15:0}	8.7	5.0
C _{15:0} 2-OH	1.0	—
iso-C _{15:0} 3-OH	5.4	3.5
iso-C _{15:1}	5.0	7.8
anteiso-C _{15:1}	5.4	5.4
C _{16:0}	2.7	—
iso-C _{16:0}	1.4	1.7
C _{16:0} 3-OH	—	1.0
iso-C _{16:0} 3-OH	2.1	2.5
C _{16:1ω5c}	—	1.0
iso-C _{17:0} 3-OH	7.7	5.3
C _{18:0}	2.4	—
Summed feature 3*	6.2	—

*Summed features are combinations of fatty acids that cannot be separated by the MIDI system. Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1ω7c}.

Fig. 1. Neighbour-joining tree showing the phylogenetic position of strain R2A-16^T, *C. normanense* CCUG 46293^T and other reference strains based on 16S rRNA gene sequences. Bootstrap values, expressed as percentages of 1000 replications, greater than 50% are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

algorithms (available as Supplementary Fig. S1 in IJSEM Online).

The above-mentioned chemotaxonomic features, together with the morphological, physiological and biochemical characteristics, strongly support the classification of strain R2A-16^T within the genus *Cloacibacterium*. Its phylogenetic distinctiveness is sufficient to categorize strain R2A-16^T as a representative of a novel species within the genus *Cloacibacterium* (Allen *et al.*, 2006). On the basis of the results obtained, strain R2A-16^T is sufficiently distinct from *C. normanense* to be recognized as a representative of a novel species of the genus *Cloacibacterium*, for which the name *Cloacibacterium rupense* sp. nov. is proposed.

Description of *Cloacibacterium rupense* sp. nov.

Cloacibacterium rupense (ru.pen'se. N.L. neut. adj. *rupense* pertaining to Rupa, the name of the lake in Nepal where the type strain was isolated).

On R2A agar, cells are 0.2–0.3 × 1.0–2.0 µm, Gram-negative, non-sporulating, motile, straight rods, occurring singly, in pairs or in long chains. Colonies grown on agar medium are yellow, round, convex, entire, waxy and semi-transparent. Colony diameter is 1–2 mm after incubation for 48 h at 37 °C. Strictly aerobic. The optimum pH for growth is 7 (range pH 5.5–8.5). The optimum temperature for growth is 37 °C (range 18–37 °C). Catalase- and oxidase-positive. Negative for nitrate reduction. Starch, gelatin, urea and aesculin are hydrolysed, but casein, DNA and Tween 80 are not. Negative for indole production and methyl red and Voges–Proskauer tests. Pigment is not of the flexirubin type. Yellow carotenoid-type pigment is produced. Resistant to streptomycin (10 µg), kanamycin (30 µg), tetracycline (30 µg) and carbenicillin (100 µg); sensitive to chloramphenicol (30 µg) and ampicillin (10 µg). The following carbohydrates can be used as sole

carbon sources: D-ribose, D-mannose, L-arabinose, D-glucose, cellobiose, lactose, maltose, sucrose, D-salicin, L-rhamnose, D-galactose, starch, D-arabinose and D-xylose. D-Fructose, raffinose, inulin, D-sorbitol, inositol, melezitose, L-rhamnose, sodium gluconate, glycerol, trehalose and D-mannitol are not used. Major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{17:0} 3-OH. Predominant respiratory quinone is MK-6.

The type strain is R2A-16^T (=CGMCC 1.7656^T =NBRC 104931^T), isolated from sediment of Rupa Lake in southern Nepal. The DNA G+C content of the type strain is 33.3 mol%.

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