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Cloacibacterium caeni sp. nov., isolated from activated sludge

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Abstract

A Gram-stain-negative, facultatively anaerobic bacterium, designated B6^T, was isolated from activated sludge of a wastewater treatment plant in South Korea. Cells were oxidase- and catalase-positive and non-motile rods producing yellow carotenoid-type pigments. Growth of B6^T was observed at 20–40 °C (optimum, 37 °C) and pH 6.6–8.2 (optimum, pH 7.0) and in R2A broth supplemented with 0–1 % (w/v) NaCl (optimum, 0 %). B6^T contained iso-$C_{15:0}$ as the major fatty acid. Menaquinone-6 was detected as the sole respiratory quinone. The G+C content of the genomic DNA of B6^T was 31.5 mol%. The results of phylogenetic analysis based on 16S rRNA gene sequences indicated that B6^T formed a tight phylogenetic lineage with members of the genus *Cloacibacterium*. B6^T was most closely related to *Cloacibacterium rupense* R2A-16^T (99.0 %), *Cloacibacterium normanense* NRS1^T (98.7 %) and *Cloacibacterium haliotis* WB5^T (97.4 %), but their DNA–DNA relatedness levels were less than 42.0 %. On the basis of phenotypic, chemotaxonomic and molecular properties, it is clear that B6^T represents a novel species of the genus *Cloacibacterium*, for which the name *Cloacibacterium caeni* sp. nov. is proposed. The type strain is B6^T (=KACC 18988^T=JCM 31714^T).

The genus *Cloacibacterium* was first described by Allen *et al.* [1] as a member of the family *Flavobacteriaceae*. At the time of writing, the genus *Cloacibacterium* consists of three species with validly published names: *Cloacibacterium normanense* isolated from municipal wastewater [1], *Cloacibacterium rupense* isolated from freshwater lake sediment [2] and *Cloacibacterium haliotis* isolated from the intestinal tract of an abalone [3]. The genus *Cloacibacterium* includes Gram-negative, catalase- and oxidase-positive and rod-shaped bacteria producing yellow carotenoid-type pigments and containing menaquinone-6 (MK-6) and iso-$C_{15:0}$ as the predominant isoprenoid quinone and cellular fatty acid, respectively. In this study, a strain representing a presumably novel species belonging to the genus *Cloacibacterium* was isolated from activated sludge and its taxonomic properties were characterized using a polyphasic approach.

Strain B6^T was isolated from a wastewater treatment plant of a Korean soju manufacturer (35° 11′ 48.43″ N, 129° 07′ 21.14″ E), located in Busan, South Korea, using a previously described procedure [4] with some modifications. Briefly, a sludge sample was obtained from an aerobic tank of the wastewater treatment plant and serially diluted with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). Aliquots of each serial dilution were spread on R2A agar (BD) and incubated aerobically at 37 °C for 5 days. Colonies grown on R2A agar were transferred by streaking cells on new R2A agar to obtain pure cultures. The 16S rRNA genes from pure cultured colonies grown on R2A agar were PCR-amplified using the universal primers F1 and R13 and the resulting PCR amplicons were double-digested with *Hae* III and *Hha* I. Representative PCR products showing unique fragment patterns were sequenced using the F1 primer and their resulting 16S rRNA gene sequences were compared with those of type strains using the nucleotide similarity search program in the EzTaxon-e server [5] and a putative novel strain belonging to the genus *Cloacibacterium*, designated B6^T, was selected for further analysis of taxonomic properties. B6^T was stored at −80 °C in R2A broth (BD) containing 15 % (v/v) glycerol for long-term preservation. The type strains of *C. haliotis* (KACC 17210^T), *C. normanense* (DSM 15886^T) and *C. rupense* (NBRC 104931^T) were used as reference strains for comparison of phenotypic properties and fatty acid compositions and DNA–DNA hybridization.

The 16S rRNA gene amplicon of B6^T that was PCR-amplified using the above F1 and R13 primers was sequenced using 340F (5′-CCTACGGGAGGCAGCAG-3′), 518R (5′-ATTACCGCGGCTGCTGG-3′) and 805F (5′-GATTAGATACCCTGGTAGTC-3′) primers at Macrogen (Seoul, Republic of Korea) to obtain an almost complete 16S rRNA gene sequence (1458 nucleotides). The 16S rRNA gene sequence similarities between B6^T and closely related type

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The GenBank accession number for the 16S rRNA gene sequence of strain B6^T is KY031327.
One supplementary figure and one supplementary table are available with the online Supplementary Material.

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strains were calculated using the nucleotide similarity search program. The phylogenetic relationships between B6^T and closely related type strains were inferred based on their 16S rRNA gene sequences. Briefly, the 16S rRNA gene sequences of B6^T and closely related type strains were aligned using the fast secondary-structure aware INFERAL aligner available in Ribosomal Database Project (RDP) [6]. Phylogenetic trees based on the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms were reconstructed using the PHYLIP software (ver. 3.695, [7]) and their tree topologies were evaluated through a bootstrap analysis based on a 1000-resampled dataset. A phylogenetic tree with bootstrap values based on the maximum-likelihood (ML) algorithm was also reconstructed using RAxML-HPC BlackBox (version 8.2.4) of the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; [8]). DNA–DNA hybridization (DDH) between B6^T and *C. haliotis* KACC 17210^T, *C. normanense* DSM 15886^T and *C. rupense* NBRC 104931^T was performed using a genome-probing microarray method, as previously described [9], which was confirmed by reciprocally interchanging DDH between probe and target DNA.

The results of comparative analysis based on the 16S rRNA gene sequences revealed that B6^T was most closely related to *C. rupense* R2A-16^T, *C. normanense* NRS1^T and *C. haliotis* WB5^T with 16S rRNA gene sequence similarities of 99.0, 98.7 and 97.4 %, respectively. The results of a phylogenetic analysis based on the NJ algorithm indicated that B6^T formed a tight phylogenetic lineage with *C. rupense* R2A-16^T within the genus *Cloacibacterium* with a 100 % bootstrap value (Fig. 1). Phylogenetic trees reconstructed using the ML and MP algorithms also supported the hypothesis that B6^T formed a tight phylogenetic lineage within the genus *Cloacibacterium* (data not shown). DNA–DNA relatedness values between B6^T and the type strains of *C. rupense*, *C. normanense* and *C. haliotis* were 42.0±2.2, 14.3±2.3 and 13.6±1.9 %, respectively, which were clearly below the 70 % threshold generally accepted for species delineation [10], indicating that B6^T represented a novel species of the genus *Cloacibacterium*.

Growth of B6^T on R2A agar (BD), laboratory prepared Luria–Bertani (LB) agar, nutrient agar (NA, BD) and tryptic soy agar (TSA, BD) was tested at 37 °C for 2 days. Temperature conditions suitable for growth of B6^T were determined on R2A agar at 15, 20, 25, 30, 37, 40 and 45 °C. Salt tolerance of B6^T was tested in R2A broth with different concentrations of NaCl (0, 0.5, 1, 1.5, 2, 2.5 and 3.0 %, w/v). Growth of B6^T was evaluated in R2A broth with different pH values (5.8–8.6 at 0.4 pH unit intervals), which were prepared using 10 mM Na_2HPO_4–NaH_2PO_4 buffer. After sterilization

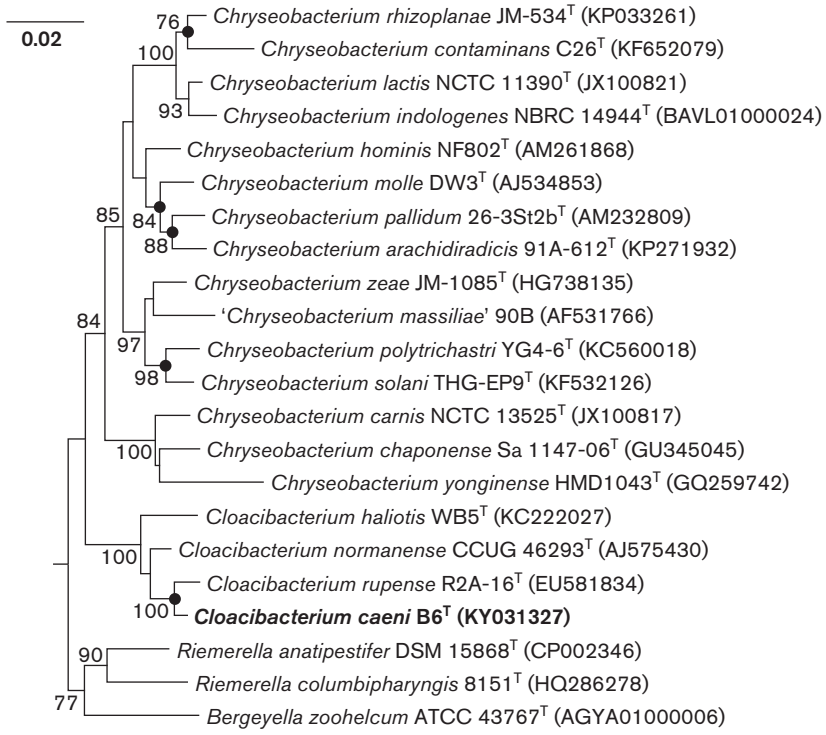

Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between B6^T and related taxa. Bootstrap values are shown at nodes as percentages of 1000 replicates; only when values are over 70 %. Filled circles (•) indicate that the corresponding nodes were also recovered in the trees generated with the ML and MP algorithms. *Polaribacter glomeratus* ATCC 43844^T (M58775) was used as an outgroup (not shown). Bar, 0.02 changes per nucleotide position.

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(121 °C for 15 min), the pH values were readjusted. Anaerobic growth was assessed on R2A agar at 37 °C for 21 days under an anaerobic condition (with 4–10 % CO_2) using the GasPak Plus system (BBL). Gram staining was tested using a Gram stain kit (bioMérieux) with cells grown on R2A agar at 37 °C for 2 days, according to the manufacturer's instructions. Catalase and oxidase activities were assessed based on bubble production with 3 % (v/v) hydrogen peroxide solution and indophenol blue production with 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), respectively. Cell morphology and motility of B6^T were investigated using transmission electron microscopy (JEM-1010, JEOL) and phase-contract microscopy (Carl Zeiss). The presence of flexirubin-type pigments was tested by flooding cells grown on R2A agar with 20 % (w/v) KOH solution [11]. To determine the production of carotenoid-type pigments, cellular pigments were extracted according to the method of Schmidt *et al.* [12] and analyzed by measuring their absorption spectra using a UV–visible spectrophotometer (Synergy MX; BioTek). Additional enzymatic activities, biochemical features and carbon source oxidation reactions of B6^T and the type strains of *C. haliotis*, *C. normanense* and *C. rupense* were investigated using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog), according to the instructions of the manufacturers.

B6^T grew on R2A agar, NA and LB agar (optimum, R2A), at 20–40 °C (optimum, 37 °C), at pH 6.6–8.2 (optimum, pH 7.0) and in R2A broth supplemented with 0–1 % (w/v) NaCl (optimum, 0 %). Anaerobic growth of B6^T was observed after 21 days of incubation on R2A agar. Cells were Gram-stain-negative and non-motile rods (0.4–0.6 µm in width and 1.1–1.4 µm in length) (Fig. S1, available in the online Supplementary Material). The absorbance spectrum of the cellular pigments extracted from B6^T had a triple-peak signature, which is a typical characteristic of carotenoid-type pigments [12]. On the Biolog GN2 MicroPlate, B6^T oxidized L-ornithine, L-proline, L-glutamic acid, L-serine, glycogen, L-aspartic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, hydroxy-L-proline and acetic acid. However, it did not oxidize other carbon sources on the Biolog GN2 MicroPlate. Physiological and biochemical characteristics of B6^T are additionally described in the species description and compared with those of members of the genus *Cloacibacterium* in Tables 1 and S1. Some of the characteristics of B6^T such as oxidase and catalase activities and carotenoid-type pigment production are in agreement with those considered to be characteristic of the members of the genus *Cloacibacterium*, whereas others, such as gelatin hydrolysis and mannose assimilation, allowed the differentiation of B6^T from the type strains of the other members of the genus *Cloacibacterium* (Tables 1 and S1).

The genomic DNA G+C content of B6^T was determined by the fluorometric method [13] using a real-time PCR thermocycler and SYBR Green I (Bio-Rad). Isoprenoid quinones of B6^T were extracted according to the method of Minnikin *et al.* [14] and analyzed using a model LC-20A HPLC system (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250×64.6 mm, Kromasil; Akzo Nobel), as described by Komagata and Suzuki [15]. Methanol/isopropanol (2 : 1, v/v) was used as an eluent (flow rate 1 ml min^{-1}) for the quinone analysis in HPLC. For cellular fatty acid analysis, B6^T and the type strains of *C. haliotis*, *C. normanense* and *C. rupense* were cultivated in R2A broth with shaking at 37 °C and their microbial cells were harvested at the same growth stage (exponential phase, OD_{600}=0.8). Cellular fatty acids of the microbial cells were saponified, methylated and extracted using the standard MIDI protocol and fatty acid methyl esters were analyzed by gas chromatography (model 6890, Hewlett Packard) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B; [16]).

The DNA G+C content of B6^T was 31.5 mol%, which was in the range of the genomic DNA G+C contents of species

Table 1. Comparisons of phenotype characteristics between B6^T and the type strains of species of the genus *Cloacibacterium*

Taxa: 1, Strain B6^T (this study); 2, *C. haliotis* KACC 17210^T [3]; 3, *C. normanense* DSM 15886^T [1]; 4, *C. rupense* NBRC 104931^T [2]. Colony colour in all strains is yellow. All strains are positive for the following characteristics: carotenoid pigments, catalase, oxidase, hydrolysis* of aesculin, activity* of esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase and assimilation* of D-glucose and D-maltose. All strains are negative for the following characteristics: flexirubin-type pigments, activity* of lipase (C14), α-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, arginine dihydrolase, urease and β-galactosidase and assimilation* of capric acid, trisodium citrate, adipic acid, *N*-acetyl-glucosamine and phenylacetic acid. +, Positive; –, negative; NA: not available.

Characteristic	1	2	3	4
Growth at/under:				
Temperature (°C)	20–40	15–37	18–36	18–37
pH	6.6–8.2	6–8	7–8	5.5–8.5
NaCl (%, w/v)	0–1	0–1.5	NA	NA
Anaerobic conditions*	+	–	+	–
Biochemical properties (API 20NE)*:				
Nitrate reduction	–	+	–	–
Indole production	+	–	+	+
Gelatin hydrolysis	–	+	+	+
D-Glucose fermentation	–	+	–	–
Enzyme activity (API ZYM and API 20NE)* of:				
Cystine arylamidase, trypsin	+	–	+	+
N-Acetyl-β-glucosaminidase	–	–	–	+
Assimilation (API 20NE)* of:				
Malate	–	+	–	–
Mannose	+	–	–	–
L-Arabinose, potassium gluconate	–	–	–	+
DNA G+C content (mol%)	31.5	29.6	31.0	33.3

*These analyses were conducted under the same conditions as used in this study.

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of the genus *Cloacibacterium* (Table 1). Menaquinone-6 (MK-6) was detected from B6^T as the sole respiratory quinone, in agreement with respiratory quinones of species of the genus *Cloacibacterium* [1–3]. The major cellular fatty acid (>10 % of the total fatty acids) of B6^T was iso-$C_{15:0}$, which was consistent with those of other species of the genus *Cloacibacterium* (Table 2). The overall fatty acid profile of B6^T was also similar to those of other species of the genus *Cloacibacterium*, although there were some evident differences in the respective proportions of some fatty acid components. For example, anteiso-$C_{15:1}$A was not detected in B6^T, but it was observed in other species of the genus *Cloacibacterium* with relatively high proportions (>8.1 %), which clearly differentiated B6^T from other species of the genus *Cloacibacterium*. Based on differences in phylogenetic, phenotypic and genotypic features of B6^T compared with those of other species of the genus *Cloacibacterium*, we suggest that B6^T represents a novel species of the genus *Cloacibacterium*, for which the name *Cloacibacterium caeni* sp. nov. is proposed.

Table 2. Comparisons of cellular fatty acid compositions (percentages) between strain B6^T and the type strains of species of the genus *Cloacibacterium*

Taxa: 1, Strain B6^T; 2, *C. haliotis* KACC 17210^T; 3, *C. normanense* DSM 15886^T; 4, *C. rupense* NBRC 104931^T. All data were obtained during this study. Data are expressed as percentages of the total fatty acids and fatty acids amounting to less than 0.3 % in all strains are not shown. Major components (>10.0 %) are highlighted in bold type. TR, Trace amount (<0.5 %); –, not detected.

Fatty acid	1	2	3	4
Saturated:				
$C_{16:0}$	0.9	0.9	0.9	0.9
Unsaturated:				
$C_{14:1}\omega5c$	0.5	TR	–	–
$C_{15:1}\omega8c$	1.1	0.5	0.7	1.3
anteiso-$C_{17:1}\omega9c$	1.9	0.6	0.8	–
Branched:				
iso-$C_{13:0}$	1.5	2.8	2.5	2.6
anteiso-$C_{13:0}$	TR	0.8	–	–
iso-$C_{14:0}$	6.8	4.9	1.7	1.5
iso-$C_{15:0}$	**33.5**	**41.3**	**34.6**	**38.5**
anteiso-$C_{15:0}$	8.6	10.5	11.1	6.4
iso-$C_{15:1}$F	7.6	5.8	7.0	7.5
anteiso-$C_{15:1}$A	–	8.6	8.1	9.7
iso-$C_{16:0}$	2.9	2.3	0.6	2.2
iso-$C_{16:1}$H	1.0	0.5	–	–
Hydroxy:				
$C_{12:0}$ 3-OH	2.0	1.7	–	1.5
$C_{13:0}$ 2-OH	1.0	0.6	0.7	1.1
iso-$C_{14:0}$ 3-OH	0.5	TR	–	–
$C_{15:0}$ 2-OH	0.7	0.9	1.1	0.8
iso-$C_{15:0}$ 3-OH	4.2	3.4	5.2	4.1
$C_{16:0}$ 3-OH	0.8	–	1.6	1.0
$C_{16:0}$ 2-OH	–	0.7	–	–
iso-$C_{16:0}$ 3-OH	6.7	3.5	2.3	3.2
$C_{17:0}$ 2-OH	0.7	0.6	1.1	–
iso-$C_{17:0}$ 3-OH	7.9	6.9	11.3	6.8
Summed features*:				
1	1.6	0.9	0.9	1.6
3	6.7	–	7.8	9.0

*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed features 1 and 3 comprise iso-$C_{15:1}$H and/or $C_{13:0}$ 3-OH and $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, respectively.

DESCRIPTION OF *CLOACIBACTERIUM CAENI* SP. NOV.

Cloacibacterium caeni (cae′ni. L. gen. n. *caeni* of sludge, referring to the isolation of the type strain from activated sludge).

Cells are Gram-stain-negative, facultatively anaerobic, non-motile rods (0.4–0.6 µm in width and 1.1–1.4 µm in length). Catalase- and oxidase-positive. On R2A agar after 2 days of incubation at 37 °C, colonies are round, convex and yellow, with entire margins. Growth occurs at 20–40 °C (optimum, 37 °C) and pH 6.6–8.2 (optimum, pH 7.0) and in R2A broth supplemented with 0–1 % (w/v) NaCl (optimum, 0 %). Yellow carotenoid-type pigments are produced but flexirubin-type pigments are not. Hydrolyzes aesculin, but not gelatin, Tween 40 and Tween 80. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase activities are positive, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, arginine dihydrolase, urease and *N*-acetyl-β-glucosamindase activities are negative. Indole production and assimilation of D-glucose, D-mannose and D-maltose are positive, but reduction of nitrate to nitrite and assimilation of L-arabinose, D-mannitol, potassium gluconate, capric acid, *N*-acetyl-glucosamine, adipic acid, malate, trisodium citrate and phenylacetic acid are negative. The major cellular fatty acid is iso-$C_{15:0}$. MK-6 is the sole isoprenoid quinone.

The type strain is B6^T (=KACC 18988^T=JCM 31714^T), isolated from activated sludge of a wastewater treatment plant in South Korea. The DNA G+C content of the type strain is 31.5 mol%.

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Conflicts of interest
The authors declare no competing financial conflicts of interests.

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