

Citrimicrobium luteum gen. nov., sp. nov., Aerobic Anoxygenic Phototrophic Bacterium Isolated from the Gut of a Sea Cucumber *Stichopus japonicus*

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A Gram-stain negative, yellow-pigmented, motile, pleomorphic bacterium, designated strain CBA4602^T, was isolated from the gut of the sea cucumber *Stichopus japonicus*, which was collected from Jeju Island in the Republic of Korea. In a phylogenetic analysis based on the 16S rRNA gene, strain CBA4602^T belonged to the order *Sphingomonadales* in the class *Alphaproteobacteria*. The 16S rRNA gene sequence similarity between strain CBA4602^T and '*Citromicrobium bathyomarinum*' JF-1, the most closely related strain having non-validly published name, was 98.4%, followed by 95.2–96.7% identities with sequence of the other closest strains in the genus *Erythrobacter*. Strain CBA4602^T had bacteriochlorophyll *a* and carotenoids. Strain CBA4602^T grew in 0–10% (w/v) NaCl, at 10–42°C and pH 6.0–8.0, with optimal growth in 1–2% NaCl, at 30–37°C and pH 7.0. Strain CBA4602^T was positive for catalase and oxidase activities and was able to hydrolyse gelatine and Tween 20 and 40, but not starch, Tween 80 or L-tyrosine. The G+C content of genomic DNA from strain CBA4602^T was 68.0 mol% and Q-10 was the major detected isoprenoid quinone. The polar lipids were three unidentified phospholipids, three unidentified glycolipids, and two unidentified lipids. The dominant fatty acids were anteiso-C_{15:0}, C_{16:0}, anteiso-C_{17:0} and C_{18:0}. As considering the current taxonomic status of the genus '*Citromicrobium*' and polyphasic taxonomic analyses, strain CBA4602^T represents a novel genus and species. The name *Citrimicrobium luteum* is proposed for the type strain CBA4602^T (=KACC 17668^T=JCM 19530^T).

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Keywords: *Citrimicrobium luteum*, sea cucumber, *Stichopus japonicus*, gut bacterium, polyphasic taxonomy

Introduction

The genus '*Citromicrobium*', a member of the order *Sphingomonadales* in the class *Alphaproteobacteria*, was described by Yurkov *et al.* (1999) as obligately aerobic anoxygenic phototrophic bacteria. The strain, '*Citromicrobium bathyomarinum*' JF-1, was isolated from water of a deep-sea hydrothermal vent plume on the Juan de Fuca Ridge, at a depth of approximately 2,000 m beneath the ocean surface. The following description characterizes the genus '*Citromicrobium*': pleomorphic, Gram-stain negative, obligately aerobic, coccoid to ovoid rods and motile by one polar or subpolar flagellum. The genus '*Citromicrobium*' was also known as having the bacteriochlorophyll *a* (Bchl *a*) and carotenoid pigments. However, the genus and species names of '*Citromicrobium bathyomarinum*' have not been validly published and strain JF-1 was not deposited in any culture collections. Here, we evaluate the taxonomic position of strain CBA4602^T isolated from the gut of the sea cucumber, which lives in shallow, temperate waters along the coasts of Southeast Asia and has been used for food. The goal of this study is to determine the taxonomic position of the novel strain CBA4602^T by phylogenetic analysis and biochemical and chemotaxonomic approaches.

Materials and Methods

Bacterial strains

Strain CBA4602^T was isolated from the gut sample from a sea cucumber, *Stichopus japonicus* (Echinodermata: *Holothuroidea*), which was collected from Jeju Island in the Republic of Korea. The gut of the sample sea cucumber was extracted and grained by a mixer and stored in distilled seawater at 4°C until cultivation. The extracted intestinal sample was serially diluted, inoculated onto R2A (BD, USA) plates containing 4% (w/v) sea salt and incubated under aerobic conditions at 37°C for 7 days. The colonies were re-streaked at least three times onto the same medium to obtain a pure colony. Pure colonies were transferred onto marine agar (MA; BD) and were cultivated at 37°C for 3 days. Stock culture of the isolate in marine broth (MB; BD) with 40% glycerol was preserved at -80°C.

Phylogenetic and genomic analysis

Phylogenetic analysis of the 16S rRNA gene sequence of strain CBA4602^T was performed. The genomic DNA of strain CBA4602^T was isolated by a genomic DNA extraction kit (RBC, Taiwan) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the AccPower PCR PreMix (Bioneer, Korea) and the universal bacterial 16S rRNA gene primer set of 8F and 1492R, and the amplification protocol was conducted as described previously (Roh *et al.*, 2008). The sequence fragments of the 16S rRNA gene were assembled using the SeqMan software program (DNASTAR) and comparisons of the 16S rRNA gene sequence were performed using the EzTaxon-e server (Kim *et al.*, 2012). The 16S rRNA gene sequences of strain CBA4602^T and its closely related species were aligned using SINA considering the secondary structure of the rRNA gene (Pruesse *et al.*, 2012). Phylogenetic trees were constructed based on the aligned 16S rRNA gene sequences using MEGA5 (Tamura *et al.*, 2011) with neighbour-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1981). A bootstrap analysis was performed by obtaining a consensus tree based on 1,000 randomly generated trees. The G+C content of genomic DNA was determined using HPLC, as described by Mesbah and Whitman (1989).

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CBA4602^T is KF612584.

Analysis of carotenoids and bacteriochlorophyll *a*

Carotenoids and Bchl *a* were extracted with acetone-methanol (7:2, v/v) and were detected using the method described by (Clayton, 1996) with a synergy plate reader (BioTech, USA). The *pufM* gene encoding a protein in the light reaction centre complex of aerobic anoxygenic phototrophic bacteria (Beja *et al.*, 2002), was detected by PCR amplification using primer set of *pufLF* (5'-CTKTTGACTTCTGGGTSGG-3') and *pufM750R* (5'-CCCATGGTCCAGCCAGAA-3') (Nagashima *et al.*, 1997) as described by Waidner and Kirchman (2008).

Morphological, physiological and biochemical characterization

The Gram reaction was performed according to the manufacturer's instructions of a Gram staining kit (bioMérieux, France). Growth was determined on MA, nutrient agar (NA; BD), tryptic soy agar (TSA; BD), Luria-Bertani agar (LBA; BD), R2A and R2A containing 4% (w/v) sea salt. Growth was investigated at different temperatures (4, 10, 15, 20, 25, 30, 37, 42, and 50°C), in the presence of varying NaCl concentrations (0, 1, 2, 3, 4, 6, 8, and 10%, w/v) and at pH 5.0–11.0 (intervals of 1.0 pH unit) on MA. Different pH values were adjusted using appropriate biological buffers: 10 mM 2-(*N*-morpholino) ethanesulfonic acid (for pH 5.0 and 6.0), 10 mM bis-Tris propane (for pH 7.0–9.0), and 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (for pH 10.0 and 11.0). Cell morphology and size were examined using a light microscopy (Eclipse 80i; Nikon, Japan) and a transmission

electron microscopy (SUPRA 55 VP; Carl Zeiss, Germany), as described previously (Lee *et al.*, 2013). A motility test was performed with semi-solid agar using the MB supplemented with 0.5% (w/v) agar. Biochemical analyses of strain CBA4602^T were performed after cultivation on MA at 37°C for 48 h. Catalase activity was tested by observing the evolution of gas bubbles upon addition of 3% (v/v) hydrogen peroxide solution. Oxidase activity was tested with indophenols blue production using 1% (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). Growth under anaerobic conditions was assessed after 7 days of incubation on MA at 37°C in the anaerobic chamber (Coy, USA). Air conditions of the chamber were N₂/H₂/CO₂ (90:5:5, vol.). Hydrolysis of starch, Tween 20, 40, and 80 and gelatine were tested as described by Smibert and Krieg (1994). Hydrolysis of L-tyrosine was performed as described by (Roh *et al.*, 2013). The enzymatic activities and carbon source assimilation were determined using API 20NE, API ZYM, and API 50CH strip (bioMérieux) according to the manufacturer's instructions. For using the API kit galleries, bacterial cells were suspended in the modified artificial seawater (23 g NaCl, 4.5 g MgCl₂·6H₂O, 4.4 g MgSO₄·7H₂O, 1.1 g CaCl₂·2H₂O, 2.2 g KCl, 0.15g KH₂PO₄, and 0.2 g NH₄Cl) according to Cha *et al.* (2013) and supplemented with 0.01% (w/v) yeast extract (González *et al.*, 1997). These cultures were cultivated at 37°C for 48 h. To test antibiotic sensitivity, the strain was inoculated on agar medium plates using antibiotic discs (µg/disc, unless indicated): ampicillin (100), bacitracin (0.5 IU), chloramphenicol (50), ciprofloxacin (30), erythromycin (50), neomycin (50), norfloxacin (100), novobiocin (50), penicillin G (50), and rifampicin (100).

Chemotaxonomy

The isoprenoid quinones were extracted from freeze-dried cells using chloroform/methanol (2:1, v/v) (Collins and Jones, 1981) and were identified by high performance liquid chromatography (HPLC) (UltiMate 3000; Dionex, USA) coupled to a diode array detector and an ion-trap mass spectrometer with electrospray ionization probe (HCT Ion-Trap MS; Bruker, USA) (Kaiser *et al.*, 2012). Polar lipids were extracted (Dittmer and Lester, 1964) and analyzed on a Merck silica gel 60 F₂₅₄ glass-backed plates, as described by Yim *et al.* (2014). The cellular fatty acid composition was determined with the saponification, methylation, and extraction steps described in the Sherlock Microbial Identification System (MIDI, 1999). The fatty acids were detected via gas chromatography (Hewlett Packard 6890, USA) and identified using the Microbial Identification software package (Sasser, 1990) based on the TSBA6 library.

Results and Discussion

Phylogenetic and genomic analysis

The 16S rRNA gene sequence of strain CBA4602^T was 1,409 bp in length. Strain CBA4602^T showed high similarity in the 16S rRNA gene sequence to '*Citromicrobium bathyomarinum*' JF-1, *Erythrobacter citreus* RE35F/1^T, *E. pelagi* UST081027-248^T, *E. nanhaisediminis* T30^T, and *E. seohaensis*

SW-135^T with 98.4%, 96.7%, 96.5%, 96.2%, and 96.0%, respectively. Similarity of the 16S rRNA gene sequence of strain CBA4602^T with other species of the genus *Erythrobacter* were less than 95.9%. The phylogenetic trees based on the 16S rRNA gene sequences, showed that strain CBA4602^T clustered with '*Citromicrobium bathyomarinum*' JF-1 with high bootstrap values of 99, 99 and 100% in the NJ, MP, and ML trees, respectively (Fig. 1). The G+C content of genomic DNA of strain CBA4602^T was 68.0 mol% which is similar with that of '*Citromicrobium bathyomarinum*' JF-1 (Yurkov *et al.*, 1999).

Analysis of carotenoids and bacteriochlorophyll *a*

Absorption spectrum of strain CBA4602^T showed carotenoids and Bchl *a* (Supplementary data Fig. S1), in agreement with the results of strain '*Citromicrobium bathyomarinum*' JF-1 (Yurkov *et al.*, 1999). The *pufM*-specific PCR assay confirmed for the existence of the *pufM* gene encoding the reaction centre of Bchl *a* for strain CBA4602^T. The distinctive features of the yellow carotenoids and Bchl *a* of strain CBA4602^T and '*Citromicrobium bathyomarinum*' JF-1 distinguish strain CBA4602^T from the phylogenetically closest validated species of *Erythrobacter citreus* which lacks Bchl *a* (Denner *et al.*, 2002).

Morphological, physiological, and biochemical characteristics

The colonies of strain CBA4602^T were yellow-pigmented, circular, smooth and opaque. Cells were motile by flagella and the morphology was pleomorphic (cocci to ovoid rod-shaped, 0.8–1.0 µm in width and 1.0–1.5 µm in length) (Supplementary data Fig. S2). Strain CBA4602^T was observed to grow on MA and R2A containing 4% (w/v) sea salt, and growth was obtained in the presence of 0–10% (w/v) NaCl,

at 10–42°C and pH 6.0–8.0. Optimal growth occurred with 1–2% (w/v) NaCl, at 30–37°C and pH 7.0. In the hydrolysis tests, strain CBA4602^T hydrolysed gelatine and Tween 20 and 40, but not starch, Tween 80 and L-tyrosine. Strain CBA4602^T was Gram-stain negative and catalase- and oxidase-positive. Cells of strain CBA4602^T did not grow anaerobically. Strain CBA4602^T was found to be sensitive to ampicillin, bacitracin, chloramphenicol, ciprofloxacin, erythromycin, neomycin, norfloxacin, novobiocin, and penicillin G, but was resistant to rifampicin. Detailed phenotypic data of strain CBA4602^T are presented in the species description and strain CBA4602^T is distinguishable from '*Citromicrobium bathyomarinum*' JF-1 and the type species in the closely related genera, based on comparison of its phenotypic characteristics (Table 1).

Chemotaxonomy

The ubiquinone detected in strain CBA4602^T was Q-10. The polar lipids of strain CBA4602^T were three unidentified phospholipids, three unidentified glycolipids, and two unidentified lipids (Fig. S3). While phosphatidylethanolamine was detected in the genera *Erythrobacter* and *Altererythrobacter* (Kumar *et al.*, 2008; Xu *et al.*, 2010; Fan *et al.*, 2011; Jung *et al.*, 2012; Wu *et al.*, 2012, 2014; Xue *et al.*, 2012; Jeong *et al.*, 2013; Subhash *et al.*, 2013; Yoon *et al.*, 2013), no aminolipids were detected in strain CBA4602^T. The dominant fatty acids in strain CBA4602^T were anteiso-C_{15:0} (24.4%), C_{16:0} (20.3%), anteiso-C_{17:0} (12.1%), and C_{18:0} (11.8%), and the minor fatty acids were anteiso-C_{19:0} (6.9%), summed feature 1 (C_{18:1ω7c} and/or C_{18:1ω6c}) (5.3%), iso-C_{17:0} (4.5%), iso-C_{15:0} (3.3%), iso-C_{19:0} (3.1%), and C_{10:0} (2.4%). The composition of major fatty acids of strain CBA4602^T (anteiso-C_{15:0} and C_{16:0}) was different with the species of the genus *Erythrobacter*, *Erythromicrobium*, *Porphyrobacter*, and *Altererythro-*

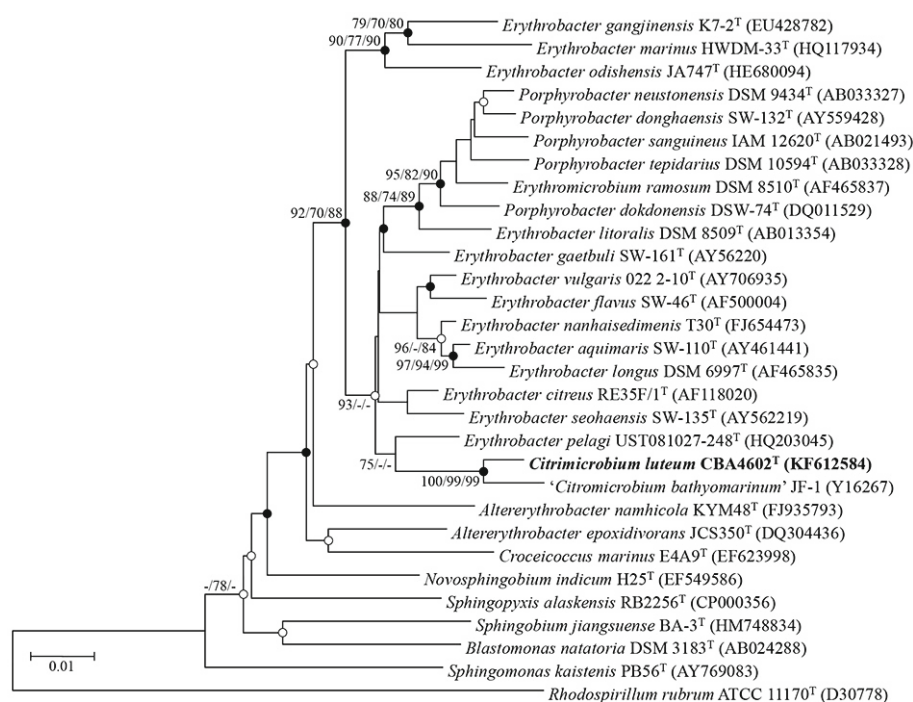


Fig. 1. Phylogenetic tree derived from the 16S rRNA gene sequences of strain CBA4602^T and the strains of closely-related species, based on the NJ algorithm. Closed circles represent nodes also recovered by the MP and ML methods. Open circles indicate nodes also recovered either by the MP or ML method. Numbers at nodes indicate bootstrap values (>70%) as calculated on the basis of NJ/MP/ML probabilities, expressed as percentages of 1000 replicates. *Rhodospirillum rubrum* ATCC 11170^T served as the out-group. Bar, 0.01 accumulated changes per nucleotide.

bacter (Denner et al., 2002; Yoon et al., 2004, 2005, 2006; Xu et al., 2010; Park et al., 2011; Wu et al., 2012) (Table 1).

Taxonomic conclusion

Strain CBA4602^T clustered tightly with the earlier effectively published '*Citromicrobium bathyomarinum*' of the family *Erythrobacteraceae* in phylogenetic analysis has resemblance to the '*Citromicrobium bathyomarinum*' JF-1 which is not available, as sharing major features: e.g. production of yellow carotenoids and Bchl *a*, obligately aerobic anoxygenic phototroph and pleomorphic cells. Strain CBA4602^T is distinguished from the genus *Erythrobacter* with the following characteristics. Cells of strains CBA4602^T and JF-1 are pleomorphic and contain Bchl *a*, but strains in the genus *Erythrobacter* are rod-shaped and lack Bchl *a*, except for *Erythrobacter longus* (Shiba and Simidu, 1982). Additionally, strain CBA4602^T can be clearly differentiated from the closely related genera with the chemotaxonomic features of the major fatty acid components and the absence of aminolipid. As mentioned above, the genus name '*Citromicrobium*' has not been validly published; therefore, it looks inadequate to propose a second species in the genus '*Citromicrobium*' based on the current taxonomic point of view. As considering the taxonomic status of the genus '*Citromicrobium*' and the malformed genus name because citron is not Greek, but citrum is Latin, we concluded to propose a novel genus and species with strain CBA4602^T under the corrected genus name *Citrimicrobium* from the previously proposed genus name '*Citromicrobium*' to avoid confusion in the field of bacterial taxonomy. Based on the polyphasic taxonomic analyses, strain CBA4602^T represents a novel genus and species in the family *Erythrobacteraceae*, for which the name *Citrimicrobium luteum* gen. nov., sp. nov. is proposed.

Description of *Citrimicrobium* gen. nov.

Citrimicrobium (Ci.tri.mi.cro'bi.um. N.L. neut. N. *citrum*, citron; Gr. adj. *micros*, small; Gr. n. *bios*, life; M. L. n. *Citrimicrobium*, citron-colored microbe).

The description of the genus is as given by Yurkov et al. (1999).

Table 1. Differential characteristics of strain CBA4602^T and phylogenetically related strains

Characteristics	1	2	3	4	5	6	7	8	9	10
Cell morphology	Pleomorphic	Pleomorphic	Rod	Rod	Rod	Rod	Rod	Rod, branched	Pleomorphic	Ovoid-rod
Cell size (µm)	0.8–1.0 × 1.0–1.5	0.4–0.5 × 1.0–1.2	0.5–0.6 × 2.5–3.0	0.3–0.7 × 1.0–1.5	0.6–0.8 × 1.5–4.0	0.3–0.5 × 0.5–1.7	0.6–0.9 × 2.0–4.0	0.6–1.0 × 1.3–2.5	0.4–0.6 × 0.5–2.5	0.4–0.6 × 0.6–1.25
Color of colonies	Yellow	Yellow	Orange	Yellow	Orange-yellow	Orange	Orange	Orange	Orange	Orange
Motility	+	+	-	-	-	+	-	+	-	-
Presence of Bchl <i>a</i>	+	+	-	-	-	-	-	+	+	-
Growth range of temperature (°C)	10–42	4–45	12–40	4–37	10–40	4–45	10–40	25–30	10–43	20–40
Optimum temperature (°C)	30–37	20–42	26–36	25–30	30–35	30	30–37	25	35–37	35
Growth at 0% (w/v) NaCl	+	+	-	+	-	-	-	+	w	-
Nitrate reduction	-	-	+	+	-	-	-	-	-	-
Hydrolysis of										
Starch	-	+	-	-	-	-	v	-	+	+
Tween 80	-	+	-	ND	+	+	+	-	+	+
Gelatin	+	-	-	ND	-	-	-	-	-	-
Utilization of										
Citrate	-	-	-	v(+)	-	-	-	+	-	-
D-Fructose	+	-	+	-	-	-	-	+	-	-
D-Glucose	+	w	+	v(+)	+	+	+	+	+	-
Malate	-	-	-	-	+	+	+	+	+	-
Susceptibility to penicillin G	+	-	+	-	-	-	-	-	+	+
Major fatty acids	anteiso-C _{15:0} , C _{16:0}	ND	C _{17:1} ω6c, C _{18:1} ω6c and/or C _{18:1} ω7c	C _{18:1} ω7c, C _{17:1} ω6c	C _{18:1} ω7c, C _{17:1} ω6c	C _{18:1} ω7c, C _{16:1} ω6c and/or C _{16:1} ω6c	C _{18:1} ω7c, C _{17:1} ω6c	C _{18:1} ω7c, C _{17:1} ω6c	C _{18:1} ω7c, C _{17:1} ω6c	C _{18:1} ω7c, C _{16:1} ω7c
Genomic G+C content (mol%)	68	67.5	60.4	62.0–62.4	62.2	59.5	62.2–62.9	63.6–64.2	65.8	54.5

Taxa: 1, *Citrimicrobium luteum* sp. nov. CBA4602^T (data from this study); 2, '*Citromicrobium bathyomarinum*' JF-1^T (Yurkov et al., 1999); 3, *Erythrobacter plagi* UST081027-248^T (Wu et al., 2012); 4, *E. citreus* RE35E/1^T (Denner et al., 2002); 5, *E. seohaensis* SW-135^T (Yoon et al., 2005); 6, *E. nanhaisediminitis* T30^T (Xu et al., 2010); 7, *E. aquimaris* SW-110^T (Yoon et al., 2004); 8, *Erythrobacter ramosum* T4^T (Yurkov et al., 1994); 9, *Porphyrobacter dokdonensis* DSW-74^T (Yoon et al., 2006); 10, *Altererythrobacter namhicola* KYW48^T (Park et al., 2011). All strains were catalase and oxidase positive and susceptible to chloramphenicol (100 µg per disc for all strains, except for *E. citreus* (30 µg)). Symbols: +, positive; -, negative; w, weak positive; ND, not determined; v, variable.

Description of *Citrimicrobium luteum* sp. nov.

Citrimicrobium luteum (lu'te.um. L. neut. adj. *luteum*, orange yellow, the colour of the colonies).

Cells are Gram-stain negative, aerobic, motile by flagella, pleomorphic (cocci to ovoid rod-shaped) and the size are 0.8–1.0 µm in width and 1.0–1.5 µm in length. The colonies are yellow, circular, smooth and opaque, with a diameter of 1–2 mm after 7 days incubation on MA at 37°C. Carotenoids, Bchl *a* and *pufM* gene are present. Growth occurs on MA and R2A containing 4% sea salt, but not on TSA, LBA, NA or R2A. Cells grow in medium containing 0–10% NaCl, at 10–42°C and at pH 6.0–8.0; optimal growth occurs in 1–2% (w/v) NaCl, at 30–37°C and at pH 7.0. Cells are catalase- and oxidase-positive. Hydrolyse gelatine and Tween 20 and 40, but not starch, Tween 80 or tyrosine. In the API ZYM test, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, cysteine arylamidase, *N*-acetyl-β-glucosaminidase, and naphthol-AS-BI-phosphohydrolase are positive, but lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase are negative. In the API 20NE test, positive for hydrolysis of esculin and gelatine and sole carbon source utilization of *N*-acetyl-glucosamine, adipate, L-arabinose, D-glucose, D-maltose, and D-mannose, but negative for the reduction of nitrate or nitrite, indole production, acid production, enzyme activities of L-arginine dihydrolase, urease or β-galactosidase and utilization of D-mannitol, gluconate, caprate, L-malate, trisodium citrate, and phenyl-acetic acid. In the API 50CH test, *N*-acetyl-glucosamine, L-arabinose, D-cellobiose, esculin, D-fructose, D-galactose, D-glucose, glycogen, D-maltose, D-mannose, and D-xylose are utilized as sole carbon sources, but D-adonitol, amygdaline, D-arabinose, D-arabitol, L-arabitol, arbutin, dulcitol, erythrol, D-fucose, L-fucose, gentiobiose, methyl-α-D-glucose, gluconate, glycerol, inulin, 2-ketogluconate, 5-ketogluconate, D-lactose, D-lyxose, D-melibiose, D-melezitose, methyl-α-D-mannoside, D-mannitol, D-raffinose, L-rhamnose, D-ribose, salicin, L-sorbose, D-sorbitol, sucrose, starch, D-tagatose D-trehalose, D-turanose, L-xylose, methyl-β-D-xyloside, and xylitol are not utilized. The major isoprenoid quinone is Q-10. The polar lipids are three unidentified phospholipids, three unidentified glycolipids, and two unidentified lipids. The dominant fatty acids are anteiso-C_{15:0}, C_{16:0}, anteiso-C_{17:0}, and C_{18:0}. The genomic DNA G+C content of strain CBA4602^T is 68.0 mol%. Type strain CBA4602^T (=KACC 17668^T =JCM 19530^T) was isolated from the gut of a sea cucumber, *Stichopus japonicus*, which was collected from Jeju in the Republic of Korea.

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