

Acetobacteroides hydrogenigenes gen. nov., sp. nov., an anaerobic hydrogen-producing bacterium in the family *Rikenellaceae* isolated from a reed swamp

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A strictly anaerobic, mesophilic, carbohydrate-fermenting, hydrogen-producing bacterium, designated strain RL-C^T, was isolated from a reed swamp in China. Cells were Gram-stain-negative, catalase-negative, non-spore-forming, non-motile rods measuring 0.7–1.0 µm in width and 3.0–8.0 µm in length. The optimum temperature for growth of strain RL-C^T was 37 °C (range 25–40 °C) and pH 7.0–7.5 (range pH 5.7–8.0). The strain could grow fermentatively on yeast extract, tryptone, arabinose, glucose, galactose, mannose, maltose, lactose, glycogen, pectin and starch. The main end products of glucose fermentation were acetate, H₂ and CO₂. Organic acids, alcohols and amino acids were not utilized for growth. Yeast extract was not required for growth; however, it stimulated growth slightly. Nitrate, sulfate, S₂O₃²⁻, thiosulfate, elemental sulfur and Fe(III) nitrilotriacetate were not reduced as terminal electron acceptors. Aesculin and gelatin were not hydrolysed. Indole and H₂S were produced from yeast extract. The G + C content of the genomic DNA was 51.2 mol%. The major cellular fatty acids were iso-C_{15:0}, anteiso-C_{15:0} and C_{16:0}. The most abundant polar lipid of strain RL-C^T was phosphatidylethanolamine. 16S rRNA gene sequence analysis revealed that the isolate belongs to the uncultured Blvii28 wastewater-sludge group (<http://www.arb-silva.de/>) in the family *Rikenellaceae* of the phylum Bacteroidetes, and shared low sequence similarities with the related species *Alistipes shahii* (81.8%), *Rikenella microfus* (81.7%) and *Anaerocella delicata* (80.9%). On the basis of these data, a novel species in a new genus of the family *Rikenellaceae* is proposed, *Acetobacteroides hydrogenigenes* gen. nov., sp. nov. The type strain of the type species is RL-C^T (=JCM 17603^T=DSM 24657^T=CGMCC 1.5173^T).

The phylum Bacteroidetes consists of four recognized classes *Bacteroidia*, *Flavobacteria*, *Sphingobacteria* and *Cytophagia* (Krieg *et al.*, 2010). The majority of strictly anaerobic species are in the order Bacteroidales of the class *Bacteroidia*. The family *Rikenellaceae* of the order Bacteroidales is a phylogenetical and phenotypical group, which contains three genera *Rikenella* (type genus), *Alistipes* and *Anaerocella*. At the time of writing, the genus *Alistipes*, comprises five species: *Alistipes putredinis*, *Alistipes finegoldii*, *Alistipes onderdonkii*, *Alistipes shahii* and *Alistipes indistinctus* (Holdeman *et al.*, 1984; Nagai *et al.*, 2010; Rautio

et al., 2003; Song *et al.*, 2006; Krieg *et al.*, 2010), which have been isolated mainly from various human clinical specimens. At the time of writing, both the genera *Rikenella* and *Anaerocella* contain only one species: strains of *Rikenella microfus* have been isolated from faecal or caecal specimens (Kaneuchi & Mitsuoka, 1978), and *Anaerocella delicata* has been isolated from a methanogenic reactor of cattle farms (Abe *et al.*, 2012). To date, all cultured members of the family *Rikenellaceae* are described as anaerobic, mesophilic, rod-shaped bacteria that usually ferment carbohydrates or proteins. Many uncultured taxonomic groups also exist in the family *Rikenellaceae*; including Blvii28, a wastewater-sludge group commonly found in anaerobic wastewater or waste treatment systems (Kaksonen *et al.*, 2004; Narihiro *et al.*, 2009; Shen *et al.*, 2013), and freshwater environments

The GenBank/EMBL/DOI accession number for the 16S rRNA gene sequence of strain RL-C^T is AB611036.

A supplementary figure is available with the online version of this paper.

(Hedlund *et al.*, 2013). Based on taxonomy (using ARB and SILVA software), the Blvii28 group contains 7952 sequences; this represents the largest 16S rRNA gene sequence number among the family *Rikenellaceae* (<http://arb-silva.de>). However, no cultivated representatives from this group have been reported so far.

In marine and freshwater environments, a high abundance of *Bacteroidetes* organisms are detected; these are considered to be of high importance for the degradation of organic matter, such as complex polysaccharides (Cottrell & Kirchman, 2000; Kirchman, 2002). In this study, the isolation of a freshwater bacterium (strain RL-C^T) belonging to the uncultured Blvii28 wastewater-sludge group in the family *Rikenellaceae* is described.

Strain RL-C^T was originally obtained from a reed swamp in Qingdao, Shandong Province, China (36° 00' 00 N 120° 00' 00 E). The medium used for isolation and cultivation of strain RL-C^T was prepared as described previously (Sekiguchi *et al.*, 2000). Decomposed reed (~1 g) from swamp was transferred into 5 ml anoxic sterile medium and vortexed. The suspension solution of rotted reed was serially diluted 5-fold in noble agar (2 %, w/v; Difco) solid roll-tubes. In reed swamp environments, microbes are considered to be responsible for the degradation of plant polysaccharides, such as cellulose, pectin and starch. A solid medium supplemented with a mixture of xylan, pectin and yeast extract in low concentrations (0.005–0.01 %, w/v) was used to imitate the natural reed swamp and thus isolate the micro-organism. The cultures were incubated at 37 °C under anaerobic condition (N₂/CO₂; 80:20, v/v). Small, light-brown and lens-shaped colonies formed after one week of incubation. The colonies were picked and transferred to a liquid medium supplemented with the mixture of xylan, pectin and yeast extract described above; growth of cells in the liquid medium was observed after 4–7 days of anaerobic incubation at 37 °C. This roll-tube isolation step (transferring single colonies from solid to liquid medium) was repeated several times until the purified strain (RL-C^T) was obtained. Strain RL-C^T was maintained on the glucose (2 mM) and yeast extract (0.01 %, w/v) medium, and growth was observed in 4–7 days of incubation at 37 °C.

Cell morphology was examined under a fluorescent microscope (BX50F; Olympus). Transmission electron microscopy (TEM) was performed with a Hitachi H-7000 transmission electron microscope as described previously (Sekiguchi *et al.*, 2003). The Gram staining reaction was performed by the method of Hucker (Doetsch, 1981). Cells of strain RL-C^T were Gram-stain-negative, non-motile rods that did not form spores (0.7–1.0 × 3.0–8.0 µm) (Fig. 1a). TEM confirmed strain RL-C^T possessed a Gram-negative-type cell wall (Fig. 1b).

The physiological characteristics of strain RL-C^T were examined as described previously (Sekiguchi *et al.*, 2000). The temperature range for growth was determined between 20 to 55 °C (at 5 °C intervals, with an exception at 37 °C).

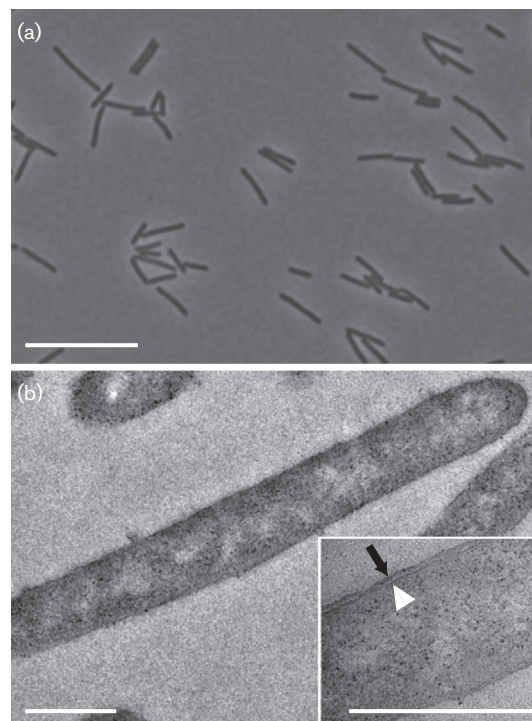


Fig. 1. Phase-contrast micrograph (a) and thin-section electron micrograph (b) of strain RL-C^T in pure culture grown on 2 mM glucose and 0.01 % (w/v) yeast extract. Triangles and arrowheads point to cytoplasmic and outer membranes, respectively. Bars, 10 µm (a) and 0.5 µm (b).

The pH range for growth was determined at 37 °C over the range pH 5.0–8.5 (in 0.5 pH unit intervals), and NaCl tolerance was tested in the presence of 0–3.0 % (w/v) NaCl (in 0.5 % intervals). The pH was adjusted at room temperature by adding HCl or NaOH to the autoclaved medium. The pH was measured at the beginning and end of the experiments. The growth of cells was evaluated based on the increase in optical density at 400 nm and the production of H₂. Short-chain fatty acids, H₂ and CH₄ were measured as described previously (Qiu *et al.*, 2014). Unless otherwise indicated, the organism was cultured anaerobically (N₂/CO₂; 80:20, v/v) at 37 °C without shaking. Aerobic growth was tested in a medium containing glucose (2 mM) and yeast extract (0.01 %, w/v) under aerobic conditions without adding reducing agents. For catalase detection test, 1 drop of 15 % (v/v) hydrogen peroxide solution was added to viable cells on a microscope slide; bubble formation indicates a catalase-positive reaction. *Methanosarcina barkeri* DSM 804 and *Sporotomaculum syntrophicum* DSM 14795^T were used as catalase-positive and -negative controls, respectively. Production of indole and H₂S, as well as hydrolysis of aesculin and gelatin, was determined according to the conventional methods described by Holdeman *et al.* (1977). Strain RL-C^T grew anaerobically on glucose/yeast extract medium at 25–40 °C (optimum, 37 °C), at pH 5.7–8.0 (optimum, approximately

pH 7.0–7.5) and with 0–1.5 % (w/v) NaCl; no growth was observed below 25 °C or above 40 °C after 8 weeks of incubation. The isolate was a strict anaerobe: it could not grow in the presence of oxygen (20 %, v/v, in the gas phase) nor after N₂/CO₂ purging (without addition of reducing agents). Growth and H₂ production were observed with the following substrates (5 mM each unless specified otherwise): arabinose, glucose, galactose, mannose, maltose, lactose, glycogen (0.1 %, w/v), pectin (0.1 %, w/v), starch (0.1 %, w/v), yeast extract (0.1 %, w/v) and tryptone (0.1 %, w/v). Xylan was weakly fermented. In the medium supplemented with glucose, the major end products were acetate, H₂ and CO₂ (1 mol of glucose was converted to approximately 1.9 mol of acetate and 5.3 mol of H₂, electron recovery: 106 %). Yeast extract was not required, however it stimulated growth slightly. Strain RL-C^T exhibited a fast growth rate; the specific growth rate (μ_{\max}) calculated was based on hydrogen production in a glucose (5 mM)/yeast (0.01 %) medium, this was estimated to be 1.8 day⁻¹. None of the following substrates tested supported the growth of strain RL-C^T in pure culture or co-culture with *Methanospirillum hungatei* DSM 864^T (5 mM unless specified): xylose, ribose, fructose, sucrose, cellobiose, melezitose, melibiose, raffinose, salicin, amygdalin, inulin (0.1 %, w/v), cellulose (0.1 %, w/v), inositol, mannitol, sodium CM-cellulose (0.5 %, w/v), Casamino acids (0.1 %, w/v), glutamate, serine, alanine, H₂/CO₂ (1 atm, 80/20, v/v) plus acetate, formate, propionate, butyrate, iso-butyrate, lactate, pyruvate, crotonate, succinate, malate, fumarate, glycerol, ethylene glycol, methanol, ethanol, 1-propanol, 1-butanol, benzoate, hydroquinone (1 mM) and phenol (1 mM). Strain RL-C^T did not use any of the following electron acceptors within 6 weeks of incubation on glucose/yeast extract medium (mM): nitrate (5), sulfate (5), sulfite (1), thiosulfate (2), elemental sulfur (5) and Fe(III) nitrilotriacetate (2). Cells were catalase-negative, aesculin and gelatin were not hydrolysed. Indole and H₂S were produced from yeast extract.

For genomic DNA G+C content determination, DNA was extracted and purified according to Kamagata & Mikami (1991). The G+C content was determined by HPLC (LC-6A; Shimadzu) with a UV detector (Shintani *et al.*, 2000). The genomic DNA G+C content of strain RL-C^T was 51.2 mol% (SD \pm 0.06 mol%). Fatty acids of cells were converted to methyl esters using HCl/methanol and identified by GC-MS (Hitachi M7200AFC/3DQMS system) (Hanada *et al.*, 2002). For fatty acid methyl ester and polar lipid analyses, cells were harvested during the exponential phase of growth from cultures anaerobically grown on 5 mM glucose and 0.02 % (w/v) yeast extract at 37 °C. The major cellular fatty acids of strain RL-C^T were iso-C_{15:0} (50.6 %), anteiso-C_{15:0} (13.2 %), C_{16:0} (11.7 %), C_{15:0} (4.8 %), iso-C_{16:0} (4.8 %), iso-C_{17:0} (4.6 %), C_{18:0} (3.7 %), anteiso-C_{17:0} (3.2 %). Polar lipids were extracted and analysed by the methods of Tindall (1990), using two-dimensional TLC (silica gel 60 F254 plates, 0.2 mm, no. 5554; Merck). The most abundant polar lipid of strain RL-C^T was phosphatidylethanolamine. Several unknown

phospholipids, aminophospholipid and polar lipids were also detected (Fig. S1, available in the online Supplementary Material).

For 16S rRNA gene sequencing, the genomic DNA of strain RL-C^T was extracted according to the method of Hiraishi (1992). 16S rRNA genes were amplified by PCR with *Taq* polymerase (Promega). The primers used in the PCR were the bacterial domain universal primer 8F and the prokaryote universal primer 1490R (Weisburg *et al.*, 1991). The PCR product was sequenced directly on a Beckman CEQ-8000 DNA sequencer using a CEQ DTC quick-start kit (Beckman Coulter). The phylogenetic tree, based on 16S rRNA gene sequences, was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA5 software package (Tamura *et al.*, 2007). The confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). A total of 1440 base pairs of the 16S rRNA gene from strain RL-C^T were sequenced and compared with other relative sequences in the GenBank and SILVA rRNA Databases Project libraries (<http://www.arb-silva.de/>). Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain RL-C^T belonged to the uncultured Blvii28 wastewater-sludge group (<http://www.arb-silva.de/>) in the family *Rikenellaceae* of the phylum *Bacteroidetes* (Fig. 2). The closest relatives to strain RL-C^T were environmental clones, such as paddy soil clone 4-CP-Fe-OTU2 (99.1 %, 16S rRNA gene sequence similarity), eutrophic bay clone C-99 (98.3 %), clone RB016 from rhizosphere of *Phragmites* (98.1 %) and clones from anaerobic granule sludge treating wastewater. In the family *Rikenellaceae*, the most closely related cultured and characterized micro-organisms were *Alistipes shahii* (81.8 %), *Rikenella microfus* (81.7 %) and *Anaerocella delicata* (80.9 %) (Song *et al.*, 2006; Kaneuchi & Mitsuoka, 1978; Abe *et al.*, 2012). Several species in the families *Porphyromonadaceae*, *Marinilabiliaceae* and *Bacteroidaceae* in the class *Bacteroidia* followed at almost the same similarities (e.g. species of the genus *Parabacteroides*, 81.6–83.1 %; species of the genus *Barnesiella*, 82.5–83.0 %, *Parabacteroides* *imshenetskii*, 82.9 %; *Bacteroides cellulosilyticus*, 81.0 %). Given the sequence similarity values for the 16S rRNA genes of strain RL-C^T and related genera were less than 83 %, we propose the creation of a novel genus and species to accommodate strain RL-C^T.

Comparative phenotypic traits of strain RL-C^T and related genera *Rikenella*, *Alistipes* and *Anaerocella* in the family *Rikenellaceae* are shown in Table 1. Strain RL-C^T shares several common phenotypic traits with related organisms in the same family, such as mesophilic, rod morphology, strictly anaerobic growth and fermentative metabolism. Strain RL-C^T differed from the related organisms by substrates range, fermentation products and isolation sources. Strain RL-C^T showed a similar carbohydrate utilization pattern to the genera *Rikenella* and *Alistipes*, whereas their fermentation products were differentiated. Species of the genera *Rikenella* and *Alistipes* ferment carbohydrates to

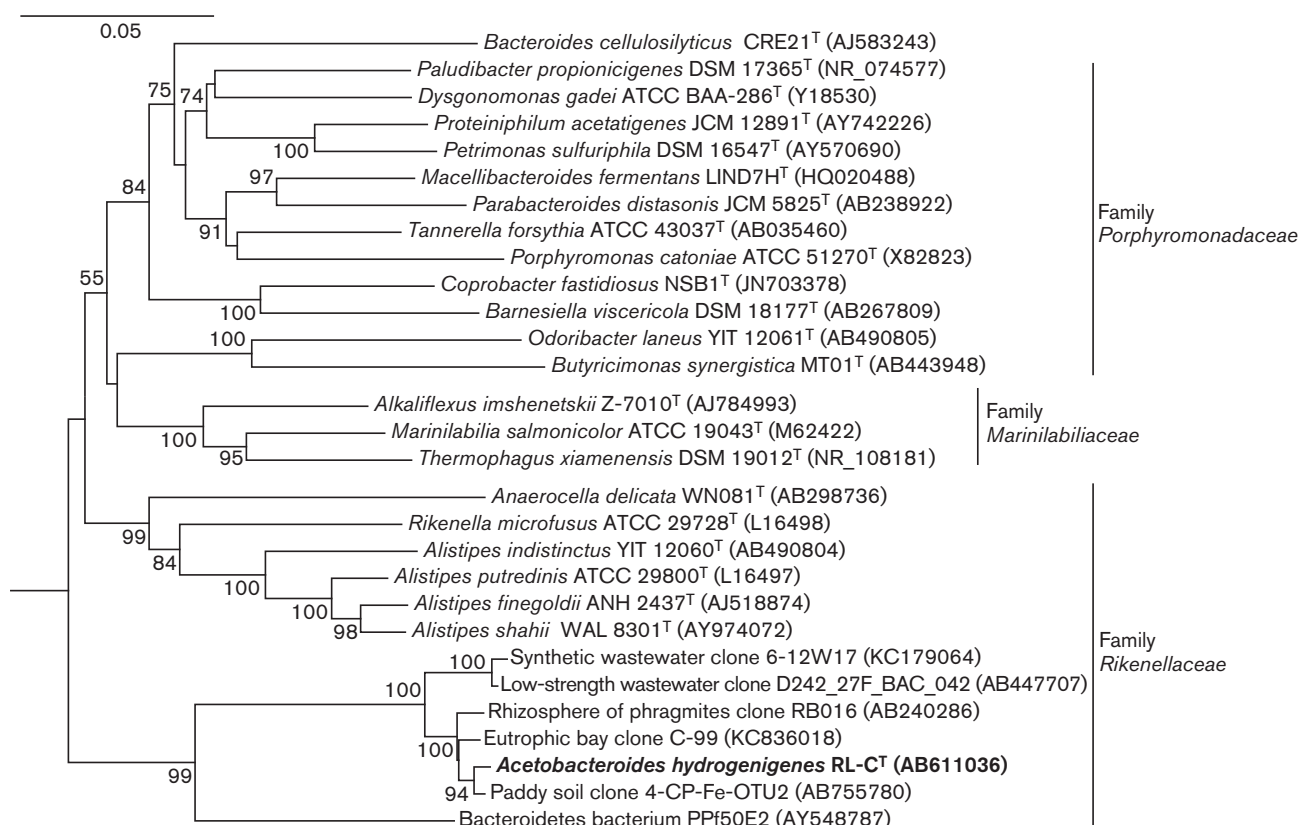


Fig. 2. 16S rRNA gene-based neighbour-joining tree showing the phylogenetic position of strain RL-C^T among the order Bacteroidales. The sequence of *Clostridium straminisolvans* DSM 16021^T (GenBank accession no. AB125279) was used to root the tree (not shown). Bootstrap values >50% from 1000 resamplings are shown at nodes. Bar, 0.05 nt substitutions per site.

complex organic acids, such as acetic, propionic, butyric, isovaleric and succinic acids, while strain RL-C^T converted carbohydrates to acetate, H₂ and CO₂ (Krieg *et al.*, 2010). Strain RL-C^T could grow on both carbohydrates and protein compounds, such as tryptone and yeast extract, however, *Anaerocella delicata* can only use proteins but not carbohydrates (Abe *et al.*, 2012). Strain RL-C^T was isolated from a reed swamp, whereas species of the genus *Alistipes* have been isolated from various human clinical specimens (Krieg *et al.*, 2010), strains of *Rikenella microfus* were isolated from faecal or caecal specimens (Kaneuchi & Mitsuoka, 1978), and *Anaerocella delicata* was isolated from a methanogenic reactor (Abe *et al.*, 2012).

On the basis of the phylogenetic, genetic, and physiological properties, it is evident that strain RL-C^T represents a novel species of a new genus in the family Rikenellaceae, for which the name *Acetobacteroides hydrogenigenes* gen. nov., sp. nov. is proposed.

Description of *Acetobacteroides* gen. nov.

Acetobacteroides [A.ce.to.bac.te.ro'i.des. L. n. *acetum* vinegar; N.L. n. *bacter* a rod; L. suff. *-oides* (from Gr. suff.

-eides, from Gr. n. *eidos* that which is seen, form, shape, figure), resembling, similar; N.L. masc. n. *Acetobacteroides* a rod-shaped micro-organism producing acetic acid].

Strictly anaerobic, Gram-stain-negative, non-motile, straight rods. Non-spore-forming. Chemo-organotrophs. Utilizes some carbohydrates for growth. Oxygen, nitrate, sulfate, sulfite, thiosulfate, elemental sulfur, and Fe(III) nitrilotriacetate do not serve as electron acceptors for growth. The genus is a member of the family Rikenellaceae.

The type species is *Acetobacteroides hydrogenigenes*.

Description of *Acetobacteroides hydrogenigenes* sp. nov.

Acetobacteroides hydrogenigenes (hy.dro.ge.ni'ge.nes. N.L. neut. n. *hydrogenium* hydrogen; Gr. *gennaio* produce; N.L. part. adj. *hydrogenigenes* hydrogen-producing, referring to the metabolic property of the species).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.7–1.0 µm in width and 3.0–8.0 µm in length. Colonies on agar are light-brown and circular shaped after cultivation at 37 °C for one week.

Table 1. Differential characteristics between strain RL-C^T and members of other related genera within the family Rikenellaceae

Strains: 1, RL-C^T (data from this study) 2, *Rikenella microfus* ATCC 29728^T (data from Holdeman *et al.*, 1984); 3, *R. sput* (Nagai *et al.*, 2010; Rautio *et al.*, 2003); 4, *Anaerocella delicata* WN081^T (Abe *et al.*, 2012). All strains are obligate anaerobes that cannot reduce nitrate. +, Positive; –, negative; (+), weak reaction.

Characteristic	1	2	3	4
Rod morphology	Straight	Spindle-shaped	Slender, straight or slightly curved with rounded ends	Straight with slightly rounded ends
Size (μm)	0.7–1.0 × 3.0–8.0	0.5–0.9 × 1.0–5.0	0.2–0.5 × 0.5–4.0	0.5–0.6 × 1.5–2.5
Optimum temperature for growth (°C)	37	37	37	25–30
DNA G + C content (mol%)	51.2	59.5–60.7	55.0–58.0	32.3
Major cellular fatty acids	iso-C _{15:0} (51 %)	iso-C _{15:0} (36 %)	iso-C _{15:0} (15–52 %)	iso-C _{15:0} (70 %)
Carbohydrates fermentation	+	(+)	+	–
Fermentation products*	Acetic acid, H ₂	Propionic, succinic acids, with moderate amounts of acetic acid and trace amounts of alcohols	Succinic acid with minor amounts of acetic acid	Acetic, propionic, isobutyric, butyric, isovaleric acids, H ₂
Isolation source	Reed swamp	Faecal or caecal specimens from calves, chickens and Japanese quails	Human and animal specimens of intestinal origin	Methanogenic reactor

*The major end products by all strains are from glucose fermentation, except *Anaerocella delicata* WN081^T from peptone/yeast extract/vitamins medium.

Grows at 25–40 °C (optimum 37 °C), at pH 5.7–8.0 (optimum pH 7.0–7.5) and in the presence of 0–1.5 % (w/v) NaCl. Ferments yeast extract, tryptone, arabinose, glucose, galactose, mannose, maltose, lactose, glycogen, pectin and starch. Xylan is weakly fermented. Yeast extract is not required for growth, however it stimulates growth slightly. Fermentation products of glucose are acetate, H₂ and CO₂. Does not utilize xylose, ribose, fructose, sucrose, cellobiose, melezitose, melibiose, raffinose, salicin, amygdalin, inulin, cellulose, inositol, mannitol, sodium CM-cellulose, Casamino acids, glutamate, serine, alanine, formate, H₂/CO₂ (1 atm, 80/20, v/v) plus acetate, propionate, butyrate, iso-butyrate, lactate, pyruvate, crotonate, succinate, malate, fumarate, glycerol, ethylene glycol, methanol, ethanol, 1-propanol, 1-butanol, benzene, hydroquinone or phenol. Cells are catalase-negative, gelatin and gelatin are not hydrolysed. Indole and H₂S are produced from yeast extract. The main fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and C_{16:0}. The most abundant polar lipid is phosphatidylethanolamine.

The type strain is RL-C^T (=JCM 17603^T=DSM 24657^T=CGMCC 1.5173^T), isolated from a reed swamp in China. The genomic DNA G+C content of the type strain is 51.2 mol%.

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