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SHORT COMMUNICATION

Clostridium brassicae sp. nov., A Strictly Anaerobic Bacterium Isolated from High-Salt Industrial Wastewater

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Abstract

An obligately anaerobic, Gram-positive, rod-shaped bacterium (1.8–5.5 µm long, 0.6–0.9 µm wide), designated ZC22-4^T, was isolated from a pickle-processing wastewater treatment plant in Zhejiang province, P.R. China. Strain ZC22-4^T grows optimally at 37–40 °C and pH 7.0 in the presence of 1% (w/v) NaCl or 2.0% (w/v) sea salts. It contained $C_{16:0}$ (25.9%), $C_{14:0}$ (13.6%), and $C_{16:1}$ *cis* 9 (10.6%) as the dominant cellular fatty acid (> 10%). Polar lipids include phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), one unidentified phospholipid (PL), two unidentified glycolipids (GL), three unidentified amino phosphoglycolipids (APGL1-3), one unidentified aminoglycolipid (AGL), and one unidentified lipid (L). The genomic DNA G + C content of ZC22-4^T was 28.7%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain ZC22-4^T belonged to the genus *Clostridium* and formed a clade with the most closely related *Clostridium aestuarii* HY-45-18^T (96.3%), *Clostridium ganghwense* HY-42-06^T (95.9%). The average nucleotide identity and DNA–DNA hybridization values among the genomes of strain ZC22-4^T and *C. aestuarii* HY-45-18^T and *C. ganghwense* HY-42-06^T were 75.7% and 77.3%, 21.7% and 23.0%, respectively. Based on the phenotypic, phylogenetic, and genetic data, strain ZC22-4^T represents a novel species in the *Clostridium* cluster I, for which the name *Clostridium brassicae* sp. nov. is proposed. The type strain is ZC22-4^T (= MCCC 1K07510T = JCM 35370^T).

Introduction

The genus *Clostridium* was established in 1880 by Prazmowski [1], the type species is *Clostridium butyricum*. As of today, *Clostridium* has become one of the largest genera of prokaryotes. 327 species were comprised in the *Clostridium* at the time of writing (https://lpsn.dsmz.de/genus/clostridium, accessed Mar. 2023), but phylogenetically many fall within cluster I (*Clostridium* sensu stricto) of the clostridia as defined by Collins et al. [2]. Most members of this genus are Gram-positive rods, represent low genomic DNA G + C, form oval or spherical endospores, usually catalase-negative [3].

Zhacai is a household name in China as a pickled vegetable. Microbial degradation has proven to be an effective technology for treating industrial wastewater [4]. During the diversity analysis of microorganisms of a pickle-processing wastewater treatment plant in Zhejiang province, PR China, a novel strain (designated ZC22-4^T) was isolated. The genomic and 16S rRNA gene sequence analysis showed that strain ZC22-4^T was closely related to members of the genus *Clostridium*. Through the study in this paper, we determined

Repositories The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZC22-4T is OR251515. The GenBank/EMBL/DDBJ accession number for draft genome sequence of strain ZC22-4T, Clostridium aestuarii HY-45-18T, and Clostridium ganghwense HY-42-06T are JAPQFJ000000000, JAPQER000000000, JAPQES000000000, respectively.

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the exact taxonomic position of strain ZC22-4^T using phylogenetic analysis.

Materials and Methods

Isolation and Cultivation

Strain ZC22-4^T was isolated from a pickle-processing wastewater treatment plant in Zhejiang province, P.R. China. Salinity, temperature, pH, and dissolved oxygen at the sampling site were measured in situ at 3.7%, 25 °C, 4.2, and 8500 mg/L, respectively.

Samples were stored in sealed sampling bottles and spiked with 0.04% cysteine, quickly transferred samples to 4 °C refrigerator for storage. 1 ml of the wastewater sample was taken and gradient diluted to 10^{-3} using 9 ml of reinforced clostridial medium (RCM), and 50 µ of the sample was spread on RCM agar plates. The RCM contained the following composition:10.0 g l^{-1} beef extract, 3.0 g l^{-1} yeast extract, 10.0 g l^{-1} peptone, 1.0 g l^{-1} soluble starch, 5.0 g l^{-1} glucose, 5.0 g l^{-1} NaCl, 3.0 g l^{-1} sodium acetate, 1 mg l^{-1} resazurin, and 0.4 g l^{-1} cysteine. For the RCM agar plates, 15.0 g l^{-1} agar was added. The medium was purged with N_2 gas to achieve an anaerobic environment. After 15 days of incubation in an anaerobic incubator (Coylab Vacuum Airlock S/N) containing a gas phase of $N_2/H_2/CO_2$ (80: 10: 10%, by vol.), strain ZC22-4^T were picked. After several rounds of streaking, purified strains were obtained and preserved in 20% glycerol (v/v) supplemented with L-cysteine (0.04%, w/v).

Strain ZC22-4^T has been deposited at the Marine Culture Collection of China (MCCC) and the Japan Collection of Microorganisms (JCM) under the conservation numbers MCCC 1K07510 and JCM 35370, respectively.

Phylogenetic Analysis Based on 16S rRNA Gene Sequences

Genomic DNA was extracted using the Quick Bacteria Genomic DNA Extraction Kit (Dongsheng Biotech; Guangzhou, P.R. China), and 16S rRNA gene was amplified by PCR with universal primers 27F (5′- AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTAC CTTGTTACGAC-3′) [5]. The PCR products were sent to Tsingke Biotechnology Co., Ltd. (Hangzhou, P.R. China) for sequencing, and then the almost-complete 16S rRNA gene sequence (1475 bp) was obtained. It has been deposited in GenBank under the number of OR251515.

The 16S rRNA gene sequences of closely related strains were obtained from the EzBioCloud database (https://www.ezbiocloud.net/) [6], and multiple sequence alignment was performed with the software Mega X [7] using the neighbor-joining(NJ) [8], maximum-likelihood (ML) [9], and maximum-evolution (ME) [8] methods. Genetic distances were calculated using the Kimura two-parameter model [10], and the tree topologies were evaluated by bootstrap analysis based on 1000 replicates.

Morphological and Physiological Analysis

In order to unify the media, the modified RCM medium (RCM supplemented with 3% (w/v) sea salts) was used to perform physiological and biochemical experiments on strain ZC22-4^T and the reference strains.

The morphological and physiological characteristics of ZC22-4^T were examined after incubation on modified RCM agar medium at 37 °C for 2 days. Transmission electron microscopy (JEM-1230, JEOL) was used to observe cell morphology, flagella, and spore after 2 and 14 days of incubation on plates. Gram staining was performed according to the method described by Buck et al. [11]. H_2S production was tested according to Wu et al. [12]. Add elemental sulfur (1%, w/v), thiosulfate (20 mM), sodium sulfite (2 mM), sodium sulfate (20 mM), sodium nitrite (2 mM), sodium nitrate (20 mM), and $Fe(OH)_3$ (10 mM) separately to the modified RCM medium separately for analyses of electron acceptors, and tested using the method of Ogg et al. [13].

Aerobic growth was tested by inoculating into aerobic medium for 14 days to observe the growth of the strain. Catalase activity was tested by using 3% (v/v) H_2O_2 to observe bubbles and oxidase activity was determined by the use of oxidase reagent (bioMérieux) [14]. The growth temperature range was tested in broth cultures of the basal medium at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45, and 50 °C. Tolerance to NaCl was detected in RCM supplemented with 0–10% (w/v) NaCl concentrations at 37 °C. Tolerance to sea salts was detected in RCM supplemented with 0–15% (w/v) sea salts concentrations at 37 °C. The pH range for growth was determined using the media from pH 5.0 to 9.5 with an interval of 0.5 pH unit. MES (for pH 5.0–6.5), MOPS (pH 6.0–7.5), Tricine (pH 8.0–8.5), and CAPSO (PH 9.0–10.0) were added at a concentration of 25 mM to maintain a stable pH. UV spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences) was used to measure the absorbance of liquid test tubes after 3 days, and growth limits were tested after 14 days of incubation at 600 nm.

Enzymatic activities, substrate utilization, and other physiological and biochemical traits were tested using API 20A strips and API 32A (bioMérieux) and the Biolog AN Microplate system according to the manufacturer's instructions. All API and Biolog tests were done in triplicate, along with reference strains.

Chemotaxonomic Characteristics

Polar lipids were extracted from 100 mg freeze-dried cell material and separated via two-dimensional silica gel TLC on silica gel 60 F254 plates (Merck) which were dried for 30 min at 55 °C before use. The total lipids were tested after spraying with molybdophosphoric acid, and the identification was then performed according to the method of Minnikin et al. [15, 16]. Isoprenoid quinones were extracted using chloroform: methanol (2:1, v/v) and identified using high-performance liquid chromatography-mass spectrometry system (Agilent 1200 and Thermo Finnigan LCQ DECA XP MAX mass spectrometer) [15]. The logarithmic phase cells of ZC22-4^T and reference strains were harvested in modified RCM after 2 days, then centrifuged and freeze-dried to analyze the fatty acids of whole cells. The fatty acids were extracted and analyzed with reference to the standard MIDI protocol (Sherlock version 6.0; MIDI database: ANAER6).

Genome Sequencing and Data Mining Analysis

The whole genome of strain ZC22-4^T was sequenced by Illumina HiSeq2500 platform by Novogene Biotech Co., Ltd (Beijing, PR China). Clean data were spliced by ABySS version 1.5.2 [17]. Splicing quality was assessed by CheckM version 1.2.0 [18].

The Type (Strain) Genome Server (https://tygs.dsmz.de/) was applied to obtain a phylogenetic tree based on the whole genome [19]. Genomic relatedness of nucleotide sequences among strain ZC22-4^T and other reference strains was determined by digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI). The dDDH values were calculated by the DSMZ Genome-to-Genome Distance Calculator (GGDC 3.0; https://ggdc.dsmz.de/ggdc.php#) with the recommended local comparison tools (blast + and Eq. 2) [20]. The ANI values were determined in the JSpeciesWS Online Service version 3.9.7 (https://jspecies.ribohost.com/jspeciesws/#analyse) [21]. The online tool BlastKOALA (https://www.kegg.jp/blastkoala/) [22] was used to reveal the metabolic pathways. Protein-encoding regions were identified with the Rapid Annotations using Subsystem Technology (RAST) [23] server. Functional annotation was performed using eggNOG-Mapper version 2.1(http://eggnog-mapper.embl.de/) [24].

Results and Discussion

Phylogenetic Relationship

Strain ZC22-4^T showed the highest 16S rRNA gene sequences similarities with *Clostridium aestuarii* HY-45-18^T (96.3%), *Clostridium ganghwense* HY-42-06^T (95.9%), and all others are below 95.9%.

The phylogenetic tree using the NJ method (Fig. 1) showed that strain ZC22-4^T was located in the *Clostridium* cluster I, and formed a monophyletic cluster with the genus *Clostridium*; the results were consistent in the ML and MP trees (Supplementary Fig S1 and S2). It suggested that ZC22-4^T represented a novel species of the genus *Clostridium*.

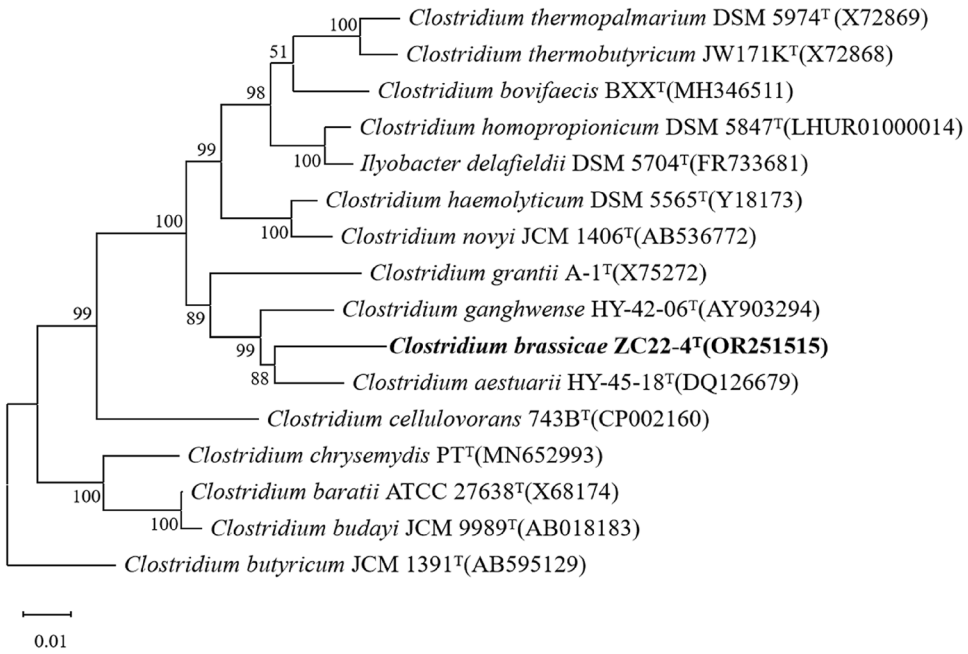

Fig. 1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain ZC22-4^T and the related species. Bootstrap values based on 1000 replicates are listed as percentages at branching points. Bar, 0.01 sequence divergence. *Clostridium butyricum* JCM 1391^T (AB595129) was used as an outgroup

Morphological and Physiological Characteristics

Based on the data obtained in this study, strain ZC22-4^T exhibited some typical characteristics of the genus *Clostridium*, such as obligately anaerobic, Gram-positive, spore-forming, rod-shaped (1.8–5.5 µm long, 0.6–0.9 µm wide, Supplementary Fig S3). However, there were still many differential characteristics to distinguish strain ZC22-4^T from the most closely related *Clostridium* species; for example, its growth does not dependent on sea salts, but *C. aestuarii* HY-45-18^T and *C. ganghwense* HY-42-06^T do not grow on RCM containing 0–5% (w/v) NaCl alone. The other phenotypic features of strain ZC22-4^T are listed in the species description and in Table 1, S1.

Chemotaxonomic Characteristics

The main fatty acids (> 10%) of strain ZC22-4^T were $C_{16:0}$ (25.9%), $C_{14:0}$ (13.6%), and $C_{16:1}$ *cis* 9 (10.6%), which are similar to those of its phylogenetic neighbors but the proportions varied. While the strains *C. aestuarii* HY-45-18^T and *C. ganghwense* HY-42-06^T had $C_{16:1}$ *cis* 7 as major fatty acids with 11.1% and 10.8%, respectively, but the content is low in strain ZC22-4^T (3.6%). The details of the fatty acid composition of strain ZC22-4^T and its closely phylogenetic neighbors were shown in Supplementary Table S2.

The strain ZC22-4^T contained phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), one unidentified phospholipid (PL), two unidentified glycolipids (GL), three unidentified amino phosphoglycolipids (APGL1-3), one unidentified aminoglycolipid(AGL), and one unidentified lipid (L) (Supplementary Fig S4). The polar lipid composition of strain ZC22-4^T and the other two strains had similarities. They all had PG, DPG, PE, PL, and GL, but APGL and AGL were unique to strain ZC22-4^T. Compared with its closely phylogenetic neighbors, ZC22-4^T contained no APL. There is no respiratory quinone was detected in strain ZC22-4^T.

Table 1 Differential phenotypic characteristics between strain ZC22-4^T and closely related members

Characteristic	1	2	3
Cell size (µm)	1.8–5.5×0.6–0.9	2–4×0.7–0.8[a]	4–8×0.7–0.8[b]
Colony pigmentation	White	Yellow	Yellow
Sea salts requirement	−	+	+
Temperature range (optimum) for growth (°C)	15–45 (37–40)	15–30 (30)[a]	15–40 (35)[b]
Sea salts range (optimum) for growth (%, w/v)	0–12(2)	1–10 (4)[a]	1–9 (3)[b]
PH range (optimum) for growth	5.0–9.0 (7.0)	5.5–8.5[a] (7.0)[a]	5.5–10.0[b](7.5)[b]
Acid produce (API 20A)			
Mannitol、lactose、maltose、glycerol、sorbitol	+	−	−
Sucrose	+	+	−
Cellobiose	+	−	+
Aesculin			
Enzymes activities (API 32A)			
α- glucosidase	+	−	+
β-glucosidase、alkaline phosphatase	−	+	+
Histidine arylamidase、serine arylamidase	+	−	−
Mannose fermentation			
Raffinose fermentation	W	−	−
Carbon source utilization(Biolog AN microplate system)			
N-acetyl-D glucosamine、L-fucose、D-galacturonic acid	+	−	+
D-cellobiose、D-gluconic acid、maltose	+	−	−
D-fructose、gentiobiose、palatinose、L-rhamnose	−	−	+
D-mannitol	+	+	-
D-melibiose	−	+	+
α-ketobutyric acid	−	+	−

Strains: 1, strain ZC22-4^T; 2, *Clostridium aestuarii* HY-45-18^T; 3, *Clostridium ganghwense* HY-42-06^T

All data were obtained from this study, except where indicated otherwise. All strains can produce H_2S, oxidase-negative and catalase-negative. Elemental sulfur, thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite, sodium nitrate, and $Fe(OH)_3$ could not be utilized as terminal electron acceptors. In the API 20A test strips, all strains are able to produce acid from glucose, sucrose, gelatin and mannose. All can utilize α-D-glucose, D-mannose, sucrose

[a]Data from Kim et al.[26]; [b]Data from Kim et al.[27]

Genome Sequencing and Data Mining Analysis

The draft genome sequence of ZC22-4^T has 4,097,941 bp, size 3.9 Mb. The genome sequence was uploaded to NCBI with the registration number JAPQFJ000000000. The genome was obtained with 71 contigs, the value of N50 was 167,691 bp, the genome coverage was 128×. The genome completeness of strain ZC22-4^T was 98.1%, with a contamination percentage of 0.6%, and was considered as an excellent reference genomes for deeper analyses [18]. The genomic DNA G+C content of ZC22-4^T was 28.7% and was similar to other two reference strains.

The ANI and dDDH values among the genomes of strain ZC22-4^T, *C. aestuarii* HY-45-18^T, and *C. ganghwense* HY-42-06^T were 75.66% and 77.33%, 21.7%, and 23.0%, respectively. All these values are well below the defined threshold for species delineation [20, 25]. The phylogenetic tree of the genome (Supplementary Fig S5) showed that strain ZC22-4^T was located in *Clostridium* cluster I but separated from other strains, hence, confirming the distinct taxonomic status of ZC22-4^T within the genus *Clostridium*.

The OrthoVenn2 online server was used to perform comparative protein sequence analysis (https://orthovenn2.bioinfotoolkits.net/home). Strain ZC22-4^T had 2502 gene clusters (Fig. S6). The three strains had 1962 gene clusters in common. Compared with the other two *Clostridium* strains, strain ZC22-4^T had 56 unique gene clusters (Supplementary Fig. S6) and 101 unique gene (Supplementary Table S3). These genes are mainly responsible for replication, recombination and repair; coenzyme transport and metabolism; carbohydrate transport and metabolism; energy production and conversion; and unknown function.

Comparative analysis of the subsystem category distribution of strain ZC22-4^T and related type strains was performed based on the genomic annotation by RAST. As shown in Supplementary Table S4, strain ZC22-4^T and the other two reference strains showed a generally similar distribution pattern of subsystem categories. For example, they possessed a large number of genes related to "cofactors, vitamins, prosthetic groups and pigments," "Nucleosides and Nucleotides," "protein metabolism," "respiration," "amino acids and derivatives," and "carbohydrates." However, strain ZC22-4^T owned more genes related to "Virulence, Disease and Defense," "Prophages, Transposable elements, Plasmids," "Iron acquisition and metabolism," "RNA Metabolism," "Regulation and Cell signaling, DNA Metabolism," "Dormancy and Sporulation," "Metabolism of Aromatic Compounds." Notably, neither *C. aestuarii* HY-45-18^T nor *C. ganghwense* HY-42-06^T contains Iron acquisition and metabolism. However, the annotation in strain ZC22-4^T obtained that periplasmic-binding protein *TonB* involved in hemin transport. It suggests that ZC22-4^T may be pathogenic. In addition, ZC22-4^T can be used as a potential research strain of *TonB* to further understand the nutrient transport process and the pathogenic mechanism of bacteria.

The differences in the KEGG metabolic pathways of the genome of strains ZC22-4^T and its reference strains were showed in Supplementary Table S5.

Description of *Clostridium brassicae* sp.nov.

Clostridium brassicae (bras.si'ca.e. L. gen. n. *brassicae* of cabbage, referring to the ingredients of pickle, from which the type strain was isolated).

Cells are Gram-positive, obligately anaerobic, oxidase-negative, catalase-negative, motile by peritrichous flagella, spore forming, rod shaped with 0.6–0.9 μm wide and 1.8–5.5 μm long. After 2 days of growth at 37 °C on RCM plates, the colonies were white with a raised center, 1.0–5.0 mm in diameter. It can be grown at 15–45 °C (optimum 37–40 °C), in 0–9% NaCl (optimum 2%) and pH 5.0–9.0 (optimum 7.0). H_2S can be produced. Elemental sulfur, thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite, sodium nitrate, and Fe $(OH)_3$ could not be utilized as terminal electron acceptors. In assays using the API 20A system, acids are produced from glucose, mannitol, lactose, sucrose, maltose, gelatin, aesculin, cellobiose, glycerol, mannose, and sorbitol. In API 32A tests, it is positive for α-glucosidase, mannose fermentation, raffinose fermentation, histidine arylamidase, serine arylamidase. According to the Biolog AN MicroPlate test, it can utilize N-acetyl-D-glucosamine, D-cellobiose, D-fucose, D-galacturonic acid, D-gluconic Acid, α-D-glucose, maltose, D-mannitol, D-mannose, and sucrose, Biolog AN MicroPlate is a method for detecting substrate utilization byanaerobic bacteria. Major cellular fatty acids are $C_{14:1}$, $C_{16:0}$, and $C_{16:1}$ *cis* 9. Polar lipids include phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), one unidentified phospholipid (PL), two unidentified glycolipids (GL), three unidentified amino phosphoglycolipids (APGL1-3), one unidentified aminoglycolipid(AGL), and one unidentified lipid (L).

The type strain is ZC22-4^T (=MCCC 1K07510T=JCM 35370^T) that was obtained from a pickle-processing wastewater treatment plant in Zhejiang province, P.R. China. The DNA G+C content of the type strain is 28.7%.

The accession number for the 16S rRNA gene sequence and draft genome sequence of strain ZC22-4^T is OR251515 and JAPQFJ000000000, respectively.

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Author Contributions MW and XYY designed the experiments and guided the manuscript writing. JYW was responsible for the major experiments, data analysis, and preparation of manuscripts. WYP and XYY assisted in enzymatic experiments. ZZW assisted in determining

fatty acids and polar lipids. YS and WWZ were involved in purchasing reference strains and revising manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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