frontiers in Marine Science

ORIGINAL RESEARCH
published: 26 August 2021
doi: 10.3389/fmars.2021.731212

Carboxylicivirga marinus sp. nov., Isolated From Marine Sediment, and Genome Insight of the Genus *Carboxylicivirga*

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A Gram-stain-negative, facultatively aerobic, beige and slender bacterium, designed N1Y132^T, was isolated from a sediment sample taken from coastal zone of Weihai, China (37°34′31.44″ N, 122°9′15″ E). Growth occurred at 15-33°C (optimal 25–28°C), at pH 6.5–8.5 (optimal pH 7.5), in 0–9% (w/v) NaCl (optimal 2.0–2.5%). According to the phylogenetic analysis based on the 16S rRNA gene sequences, strain N1Y132^T showed the highest sequence similarity (97.9%) with *Carboxylicivirga taeanensis* JCM 19490^T, followed by *C. sediminis* JR1^T (97.4%), *C. mesophila* JCM 18290^T (96.4%) and *C. linearis* FB218^T (95.1%). Thus, strain N1Y132^T was a member of the genus *Carboxylicivirga.* The average nucleotide identity (ANI) values between strain N1Y132^T and *C. sediminis* JR1^T, *C. linearis* FB218^T and *C. mesophila* JCM 18290^T were 74.8%, 71.1%, and 74.9%, respectively. Chemotaxonomic analysis showed that the sole respiratory quinone was MK-7 and the major fatty acids (> 5.0%) included iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{13:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$ 3-OH, and $C_{15:1}\omega6c$. The polar lipids were consisted of a phosphatidylethanolamine, two phosphoaminolipids and six unidentified lipids. The DNA G + C content was 38.1 mol%. Based on the evidence presented in this study, strain N1Y132^T represents a novel species of the genus *Carboxylicivirga*, for which the name *Carboxylicivirga marinus* sp. nov. is proposed. The type strain is N1Y132^T (= KCTC 72934^T = MCCC 1H00431^T).

Keywords: ***Carboxylicivirga*, 16S rRNA gene, polyphasic taxonomy, draft genome sequencing, genome analysis**

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Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Marine Science

Received: *26 June 2021*
Accepted: *05 August 2021*
Published: *26 August 2021*

Citation:
Zhang T-S, Zhang Y-N, Lu D-C, Gong Y and Du Z-J (2021) Carboxylicivirga marinus sp. nov., Isolated From Marine Sediment, and Genome Insight of the Genus Carboxylicivirga. Front. Mar. Sci. 8:731212. doi: 10.3389/fmars.2021.731212

INTRODUCTION

The genus *Carboxylicivirga*, a member of family *Marinilabiliaceae* of the phylum *Bacteroidetes*, was originally proposed by Yang et al. (2014). Bacteria of the genus *Carboxylicivirga* are Gram-stain-negative and slender, and the major respiratory quinone is MK-7. At the time of writing, 5 species are assigned to the genus, including *C. mesophila* (Yang et al., 2014), *C. taeanensis* (Yang et al., 2014), *C. linearis* (Wang et al., 2015), *C. flava* (Wang et al., 2016), and *C. sediminis* (Wang F. Q. et al., 2018). Without exception, members of the genus *Carboxylicivirga* are isolated from marine sediments. The 16S rRNA gene sequencing revealed that *Carboxylicivirga* was the core

Abbreviations: KCTC, Korea Collection for Type Culture; MCCC, Marine Culture Collection of China; JCM, Japan Collection of Microorganisms; NJ, neighbor-joining; ME, minimum-evolution; ML, maximum-likelihood; MA, marine agar 2216; MB, marine broth 2216; ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization; HPLC, High Performance Liquid Chromatography.

genera in the gut of marine invertebrates, such as the mud crab *Scylla paramamosain* (Wei et al., 2019) and the lobster *Homarus gammarus* (Holt et al., 2019). However, the function of this newly recognized genus in the environment or in the host are less known. In this study, a beige, non-motile and facultatively aerobic bacterium, strain N1Y132^T, is proposed as representing a novel species of genus *Carboxylicivirga*. Comparative analysis of genomes of strain N1Y132^T and reference strains *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T are contributed to understand the function of genus *Carboxylicivirga*.

MATERIALS AND METHODS

Bacterial Isolation and Cultivation

Strain N1Y132^T was isolated from a sediment sample taken from coastal zone of Weihai, China (37°34′31.44″ N, 122°9′15″ E), in October 2018. For the bacterial isolation, the sediment sample was homogenized and 10-fold serially diluted to 10^{-5} with sterile seawater and 100 μl aliquot of each dilution was spread on marine agar 2216 (MA; Becton Dickinson) and incubated at 28°C for 21 days. The strain, designed N1Y132^T, was incubated on MA at 28°C and was stored at −80°C in sterile 1% (w/v) saline supplemented with 15% (v/v) glycerol. The type strains *C. mesophila* JCM 18290^T (obtained from Japan Collection of Microorganisms, Japan), *C. taeanensis* JCM 19490^T (obtained from Japan Collection of Microorganisms, Japan), *C. sediminis* JR1^T (generously provided by Wang FQ, Wang F. Q. et al., 2018) and *C. linearis* FB218^T (storage in our lab) were used as reference strains and cultivated routinely on MA at 28°C.

Phenotypic, Physiological, and Biochemical Characteristics

The morphological and physiological features of strain N1Y132^T were examined after incubation at 28°C for 3 days on MA plates. Cell morphology and size were observed by light microscope (E600, Nikon) and transmission electron microscope (JEM-1200, Jeol). Gram reaction was determined using the method described by Smibert and Krieg (1994). Motility was examined on MA soft agar (0.3% agar). Gliding motility was examined according to the method described by Bowman (2000). Growth range and optimum of temperature were evaluated by observation of visible colonies on modified MA at various temperatures (0, 4, 10, 15, 20, 25, 28, 30, 33, 37, 40, and 45°C). The effects of different salt concentrations on growth were tested by using a medium comprised of 0.1% yeast extract, 0.5% peptone and 1.8% agar, prepared with artificial seawater (0.32% $MgSO_4$, 0.23% $MgCl_2$, 0.12% $CaCl_2$, 0.07% KCl and 0.02% $NaHCO_3$, all w/v) and containing different concentrations of NaCl (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12%, w/v). The pH range (from pH 5.5 to 9.5, in intervals of 0.5 pH units) and optimal pH for growth was tested in marine broth 2216 (MB) medium by using the appropriate buffer solution, including MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5), and CAPSO (pH 9.0 and 9.5). The HCl or NaOH was used to adjust the pH of the medium before autoclaving. Growth under anaerobic (10% H_2, 10% CO_2, and 80% N_2) and microaerobic (5% O_2, 10% CO_2, and 85% N_2) conditions was examined after incubation for 2 weeks on MA in an anaerobic jar with or without 0.1% (w/v) KNO_3. Catalase activity was detected by bubble production in 3% (v/v) H_2O_2. Oxidase activity was determined using an oxidase reagent kit (bioMérieux) according to the manufacturer's instructions. Antibiotic susceptibility of strain N1Y132^T was investigated on MA plates at 28°C for up to 3 days (0.5 McFarland standard) using the disc diffusion method as described previously (Du et al., 2014) and according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). Bacterial abilities to hydrolyze agar, DNA, alginate, starch, casein, CM-cellulose and Tweens (20, 40, 60, and 80) were tested according to the

TABLE 1 | Differential phenotypic characteristics of strain N1Y132^T and the related *Carboxylicivirga* species.

Characteristics	1	2	3	4	5
Cell size (μm)	0.3–0.4 × 5.0–50.0	0.3–0.4 × 2.0–15.5[a]	0.3–0.4 × 4.0–25.5[b]	0.4–0.7 × 8.2–11.6[c]	0.3–0.6 × 6.1–18.7[c]
pH range for growth	6.5–8.5 (7.5)	6.0–8.5 (7.0–7.5)[a]	6.0–9.0 (6.5–7.0)[b]	6–9 (7.0)[c]	6–8.5 (7.5)[c]
NaCl range (%)	0–9 (2.0–2.5)	0–10 (2–3)[a]	1–7 (2–3)[b]	0–8 (2.5)[c]	0–10 (2.5)[c]
Growth temperature (°C)	15–33 (25–28)	15–42 (33)[a]	15–40 (30)[b]	18.2–38.1 (30.3)[c]	15–41 (30)[c]
Catalase	−	w	+	+	+
Hydrolysis of					
Starch	+	+[a]	+[b]	w[c]	+[c]
Agar	−	+[a]	w[b]	−[c]	−[c]
Gelatin	+	+	+	+	+
Indole production	−	+	+	−	−
Acid production from					
D-ribose	+	−	−	−	−
D-xylose	+	+	+	+	−
D-glucose	−	+	w	+	+
D-fructose	w	−	+	−	−
Esculin	+	+	+	+	+
D-tagatose	w	−	−	−	−
Potassium 5-ketogluconate	+	−	−	−	w
Enzyme activity					
Alkaline phosphatase	+	+	+	+	+
Trypsin	−	+	+	+	−
Naphthol-AS-Biphosphohydrolase	w	+	+	+	+
β-galactosidase	−	+	+	−	−
N-acetyl-β-glucosaminidase	+	+	+	+	+
Oxidation of					
D-cellobiose	−	+	+	+	−
3-methyl glucose	+	−	−	−	−
L-histidine	+	−	+	−	−
D-lactic acid methyl ester	+	−	−	−	w
α-keto-glutaric acid	+	−	+	−	−
α-keto-butyric acid	−	w	+	+	−

Strains: 1, N1Y132^T; 2, C. sediminis JR1^T; 3, C. linearis FB218^T; 4, C. mesophila JCM 18290^T; 5, C. taeanensis JCM 19490^T. +, positive; w, weakly positive; −, negative. All data were obtained from this study unless otherwise indicated. Data from: a, Wang F. Q. et al. (2018); b, Wang et al. (2015); c, Yang et al. (2014).

methods from Smibert and Krieg (1994). Nitrogen fixation ability was tested by using a nitrogen free medium comprised of 0.1% starch and 1.8% agar, prepared with artificial seawater (0.32% $MgSO_4$, 0.23% $MgCl_2$, 0.12% $CaCl_2$, 0.07% KCl and 0.02% $NaHCO_3$, all w/v) at 28°C for 2 weeks in aerobic and anaerobic conditions (Wang H. B. et al., 2018). Additional physiological and biochemical characteristics were determined using API 20E and API ZYM kits (bioMérieux). Tests of acid production from carbohydrates were detected using the API 50CHB fermentation kit (bioMérieux). Oxidation of substrate was examined in Biolog GEN III MicroPlates. All API tests were performed according to the manufacturer's instructions (except for salinity, which was adjusted to 3%).

16S rRNA Gene Sequence Analysis

The 16S rRNA gene was amplified by PCR using the *Taq* DNA polymerase with the universal primers 27F and 1492R (Lane, 1991). The purified PCR product with 3′-A overhangs was cloned into a pMD18-T vector (Takara) based on the TA cloning method, and the ligation product was transformed into *Escherichia coli* DH5α cells. The positive clones were selected and sequenced bi-directionally with primers M13-47 and RV-M at BGI Co. Ltd (Qingdao, China) using the ABI 3730XL system. Thus, a near-complete 16S rRNA gene sequence (1500 bp) of strain N1Y132^T was gained. The determined 16S rRNA gene sequence for strain N1Y132^T was submitted to the National Centre for Biotechnology Information (NCBI) GenBank database. The 16S rRNA gene similarities were calculated using the EzBioCloud Database (Yoon et al., 2017). Multiple sequences were aligned using the CLUSTAL_X program (Thompson et al., 1997). In order to determine the phylogenetic position of strain N1Y132^T, phylogenetic trees were constructed using the neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-likelihood (ML) (Fitch, 1971) and maximum-parsimony (MP) (Felsenstein, 1981) methods in MEGA version 7.0 (Kumar et al., 2016). The reliability of relationships was ensured by performing bootstrap analyses based on 1000 replications.

Chemotaxonomic Characterization

In order to determine the chemotaxonomic features, the cells of strain N1Y132^T and *C. sediminis* JR1^T, *C. linearis* FB218^T, *C. mesophila* JCM 18290^T, and *C. taeanensis* JCM 19490^T were harvested by centrifuge and subjected to freeze-drying after cultivation in marine broth 2216 medium for 3 days at 28°C. Fatty acid methyl esters (FAMEs) were extracted from 30 mg freeze-dried biomass according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.1). Fatty acids were prepared and analyzed by using an Agilent 6890N gas chromatograph. Peaks were automatically integrated, and fatty acid names and percentages were determined using the TSBA40 database of the Microbial Identification system (Sasser, 1990). Respiratory quinones of strain N1Y132^T were obtained from 300 mg freeze-dried cells using the two-stage method described by Tindall et al. (2007) and analyzed by HPLC (Kroppenstedt, 1982). Polar lipids were extracted from 50 mg of freeze-dried cell material using a chloroform/methanol system according to the procedures described by Komagata and Suzuki (Komagata and Suzuki, 1988). Polar lipids were analyzed by two-dimensional thin layer chromatography according to Minnikin et al. (1984).

Genomic Analysis

Genomic DNAs of strain N1Y132^T and reference strains *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T were extracted by using a genomic DNA extraction kit (Takara) according to the manufacturer's recommendations. The draft genome sequence of N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T were sequenced on the Illumina HiSeq PE150 platform at Beijing Novogene Bioinformatics Technology (Beijing, China). The

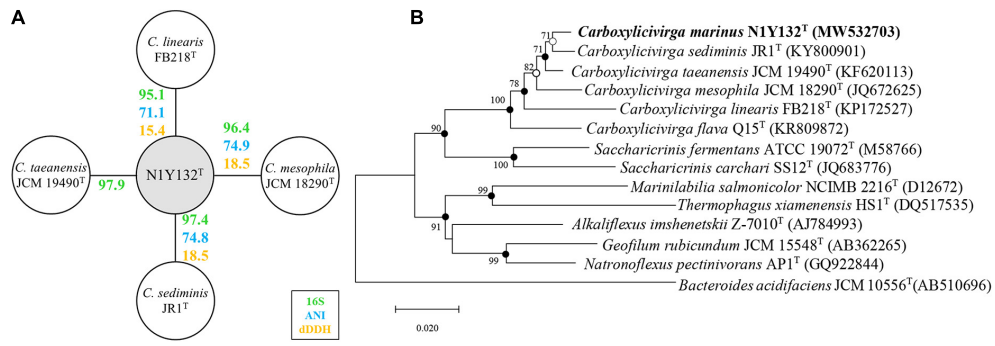

FIGURE 1 | Phylogenetic relationship of strain N1Y132^T and other members of family *Marinilabiliaceae*. **(A)** The similarities shared between strain N1Y132^T, *C. taeanensis* JCM 19490^T, *C. sediminis* JR1^T, *C. mesophila* JCM 18290^T, and *C. linearis* FB218^T for 16S rRNA gene sequence similarity (16S), average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH). The 16S rRNA gene similarities were calculated using the EzBioCloud Database. Average nucleotide identity (ANI) was calculated using ANI calculator (https://www.ezbiocloud.net/tools/ani/). The digital DNA-DNA hybridization (dDDH) were calculated using the genome-to-genome distance calculator (GGDC) website (http://ggdc.dsmz.de/distcalc2.php). **(B)** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain N1Y132^T and related species. Bootstrap values ≥70% (based on 1000 replications) are shown at branching nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in either the maximum-likelihood or maximum-parsimony algorithm. *Bacteroides acidifaciens* JCM 10556^T (GenBank accession number, AB510696) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

genome sequence was deposited in the DDBJ/GenBank/EMBL database. The G + C content of the chromosomal DNA was calculated using genome sequence. Homologous genomic regions of four *Carboxylicivirga* species were analyzed using progressiveMauve algorithm (Darling et al., 2004). Core genes and specific genes of four strains were analyzed by the CD-HIT (Fu et al., 2012) rapid clustering of similar proteins software with a threshold of 40% pairwise identity and 0.4 length difference cutoff in amino acid. Then, the Venn figure was drawn to show their relationships among the samples.

The genes of involved in N1Y132^T metabolic pathways were analyzed using the KofamKOALA (Aramaki et al., 2020[1]) and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016[2]). Carbohydrate-active enzymes of strain N1Y132^T and three reference strains were annotated using the dbCAN2 meta server (Zhang et al., 2018). The presence of gene clusters encoding secondary metabolites of strain N1Y132^T was predicted by using the antiSMASH 6.0 database (Blin et al., 2021). The average nucleotide identity (ANI) was calculated using ANI calculator (Lee et al., 2016[3]). In addition, digital DNA-DNA hybridization (dDDH) values between strain N1Y132^T and reference strains were obtained using the genome-to-genome distance calculator (GGDC) website (Meier-Kolthoff et al., 2013)[4].

[1]https://www.genome.jp/tools/kofamkoala/

[2]https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

TABLE 2 | Cellular fatty acid contents (%) of strain N1Y132^T and the related *Carboxylicivirga* species.

Fatty acid	1	2	3	4	5
Saturated					
$C_{16:0}$	2.4	**7.5**	2.3	1.5	2.4
$C_{17:0}$	1.4	TR	TR	TR	TR
Branched chain					
iso-$C_{12:0}$	–	–	TR	–	–
iso-$C_{13:0}$	**6.7**	1.8	–	3.5	3.2
iso-$C_{14:0}$	**6.6**	1.3	–	4.0	**6.5**
iso-$C_{15:0}$	**20.7**	**26.0**	**42.4**	**25.2**	**28.8**
anteiso-$C_{15:0}$	**10.2**	**10.3**	**15.8**	**9.4**	**11.4**
iso-$C_{16:0}$	4.9	3.9	4.6	3.5	2.1
iso-$C_{17:0}$	1.0	1.9	1.2	TR	1.1
Monounsaturated					
$C_{15:1}\omega6c$	**6.0**	2.8	–	4.8	4.0
$C_{16:1}\omega5c$	4.9	**7.6**	TR	**5.0**	2.4
$C_{17:1}\omega6c$	2.7	**5.4**	TR	4.1	2.4
$C_{13:1}$ AT 12-13	–	1.6	–	TR	TR
Hydroxy					
$C_{15:0}$ 2-OH	1.8	TR	1.3	1.7	1.0
iso-$C_{15:0}$ 3-OH	**6.2**	4.8	**9.2**	**8.6**	**5.8**
$C_{16:0}$ 3-OH	TR	4.1	TR	TR	TR
iso-$C_{16:0}$ 3-OH	3.1	1.5	3.1	2.1	2.0
$C_{17:0}$ 2-OH	1.0	TR	TR	TR	TR
iso-$C_{17:0}$ 3-OH	1.8	3.1	4.3	TR	3.1
Summed features*					
3	3.2	4.9	TR	2.5	3.0
4	3.9	4.3	3.4	**10.7**	**6.1**

*Strains: 1, N1Y132^T; 2, C. sediminis JR1^T; 3, C. linearis FB218^T; 4, C. mesophila JCM 18290^T; 5, C. taeanensis JCM 19490^T. All data were obtained from this study. The fatty acids in bold are the major cellular fatty acids (> 5%). Symbols: -, Not detected; TR, trace (< 1%). Values are percentages of the total fatty acids. *Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed Features 3 consisted of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ and Summed Features 4 consisted of iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B, according to Montero-Calasanz et al. (2014).*

RESULTS AND DISCISSION

Morphological, Physiological, and Biochemical Characteristics

Transmission electron microscopy showed that the cells of strain N1Y132^T were slender and non-flagellar (**Supplementary Figure 1**), which were similar with the closely related strains *C. sediminis* JR1^T, *C. linearis* FB218^T, *C. mesophila* JCM 18290^T, and *C. taeanensis* JCM 19490^T. However, cells of strain N1Y132^T were longer than these related strains (**Table 1**). Strain N1Y132^T was susceptible to clarithromycin (15 μg), ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), rifampicin (5 μg), ceftriaxone (30 μg), penicillin (10 μg), cefotaxime sodium (30 μg) and carbenicillin (100 μg), but resistant to vancomycin (30 μg), gentamicin (10 μg), polymyxin B (300 μg), ofloxacin (5 μg), norfloxacin (10 μg), lincomycin (2 μg), tetracycline (30 μg), and tobramycin (10 μg). The strain N1Y132^T was positive for the hydrolysis of starch and gelatin, but negative for agar, DNA, alginate, casein, CM-cellulose and Tweens (20, 40, 60, and 80). According to the results of commercial kits, all the five strains showed positive reactions for alkaline phosphatase, acid phosphatase, naphthol-AS-Biphosphohydrolase, N-acetyl-β-glucosaminidase and esculin, but negative reactions for esterase

[3]https://www.ezbiocloud.net/tools/ani/

[4]http://ggdc.dsmz.de/distcalc2.php

TABLE 3 | Genome statistics of strain N1Y132^T and the related *Carboxylicivirga* species.

	1	2	3	4
Genome size (bp)	5,649,616	5,808,478	6,050,047	5,650,892
N50 value (bp)	72,701	137,867	236,764	181,737
Contigs (no.)	206	133	181	87
G + C content (mol%)	38.1	40.9	36.4	41.2
Annotation by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP)				
Genes (no.)	4,463	4,590	4,842	4,372
Protein-coding genes (no.)	4,383	4,512	4,770	4,302
Hypothetical proteins (no.)	1,021	1,022	1,027	876
tRNA (no.)	59	65	59	57
rRNA (no.)	3	4	3	4
ncRNAs (no.)	3	3	3	3
Sequencing depth of coverage	110.0 ×	150.0 ×	160.0 ×	165.0 ×
DDBJ/ENA/GenBank accession number	JAENRR 000000000	JAGTAR 000000000	JAGUCO 000000000	JAGUCN 000000000

Strains: 1, N1Y132^T; 2, C. sediminis JR1^T; 3, C. linearis FB218^T; 4, C. mesophila JCM 18290^T. All data were obtained from this study.

(C4), esterase lipase (C8), α-galactosidase, H_2S production and D-adonitol. Given these positive results, the ability of strain N1Y132^T to utilize substrate was weaker than that of these three reference strains. Detailed phenotypical features were listed in species description as well as in **Table 1** and the results of commercial kits were listed in **Supplementary Table 1**.

Phylogenetic Analysis of 16S rRNA Gene Sequences

After PCR amplification and sequencing, a near-complete 16S rRNA gene sequence (1500 bp) of strain N1Y132^T was obtained and subjected to comparative analysis. BLAST searches revealed that strain N1Y132^T shared the highest 16S rRNA gene sequence similarity with *C. taeanensis* JCM 19490^T (97.9%, **Figure 1A**). In addition, strain N1Y132^T shared the 16S rRNA gene sequence similarity of 96.4% with *C. mesophila* JCM 18290^T, which was the type species of the genus *Carboxylicivirga*. In the resulting NJ tree, strain N1Y132^T was placed within the clade comprising the genus *Carboxylicivirga* and was located nearest to *C. sediminis* JR1^T (**Figure 1B**). MP and ML trees also showed similar tree topologies. Both the sequence similarities and phylogenetic relationships indicated that strain N1Y132^T may represent a novel species of the genus *Carboxylicivirga*.

Chemotaxonomic Characteristics

The major fatty acids (> 5%) of strain N1Y132^T were iso-$C_{15:0}$ (20.7%), anteiso-$C_{15:0}$ (10.2%), iso-$C_{13:0}$ (6.7%), iso-$C_{14:0}$ (6.6%), iso-$C_{15:0}$ 3-OH (6.2%), and $C_{15:1}$ ω6*c* (6.0%), which were similar in composition to those of other species in genus *Carboxylicivirga* (**Table 2**). However, some differences between strain N1Y132^T and other *Carboxylicivirga* species were observed. For example, *C. mesophila* JCM 18290^T, *C. linearis* FB218^T and *C. taeanensis* JCM 19490^T contained $C_{13:1}$ AT 12-13, which was not detected in strain N1Y132^T and *C. sediminis* JR1^T. The respiratory quinone of strain N1Y132^T was determined to be MK-7 (Kroppenstedt, 1982), which was in line with the description of the genus *Carboxylicivirga* (Yang et al., 2014). The polar lipids of strain N1Y132^T were comprised of a phosphatidylethanolamine, two phosphoaminolipids and unidentified lipids 1-6 (**Supplementary Figure 2**). Phosphatidylethanolamine and several unknown polar lipids were detected predominantly in tested *Carboxylicivirga* species, in line with the earlier report (Wang F. Q. et al., 2018).

Genome Insight of Four Strains Belonging to *Carboxylicivirga* Genus

Basic Characteristics of Four Genomes

The genome length of strain N1Y132^T, *C. mesophila* JCM 18290^T, *C. linearis* FB218^T, and *C. sediminis* JR1^T were less differentiating these four strains (**Table 3**). Among them, *C. linearis* FB218^T harbors the largest genome (6,050,047 bp, JAGUCO000000000), while the new strain N1Y132^T holds the smallest one (5,649,616 bp, JAENRR000000000). The sequencing depth of coverage and the contig numbers are different for these four draft genomes. However, some basic genome characteristics have been analyzed. The DNA G + C content of strain N1Y132^T

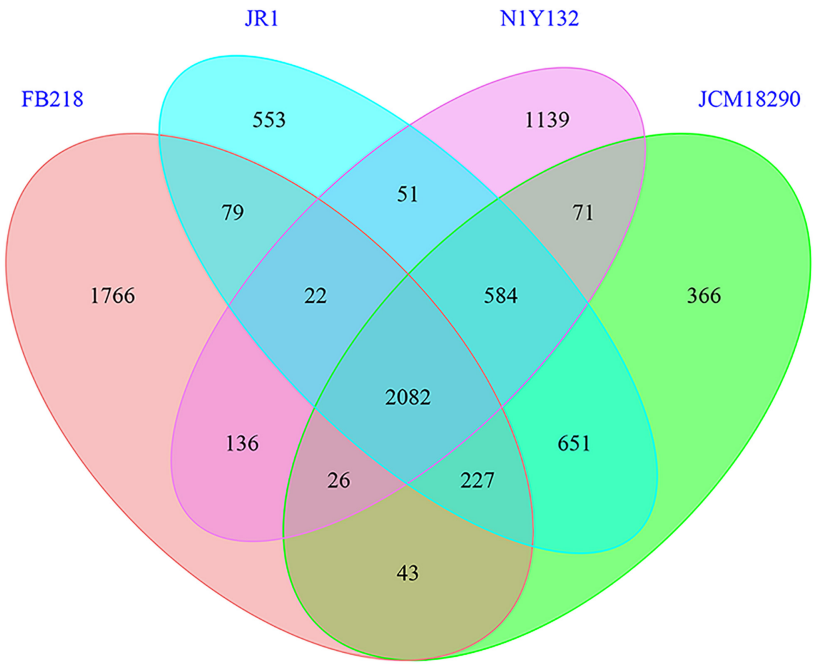

FIGURE 2 | Distribution of orthologous genes in the genomes of the four *Carboxylicivirga* species (strain N1Y132^T, *C. mesophila* JCM 18290^T, *C. linearis* FB218^T, and *C. sediminis* JR1^T) tested by the CD-HIT rapid clustering of similar proteins software. 2082 core gene clusters that were represented in all four genomes. Strain N1Y132^T, *C. mesophila* JCM 18290^T, *C. linearis* FB218^T, and *C. sediminis* JR1^T contained 1139, 366, 1766, and 553 species-specific gene clusters, respectively. The clustering results were used to generate Venn's diagram (**Supplementary Data 1**).

was 38.1 mol%, which was higher than that of *C. linearis* FB218^T (36.4 mol%), but lower than that of *C. mesophila* JCM 18290^T (41.2 mol%) and *C. sediminis* JR1^T (40.9 mol%).

A total of 4,463 genes were predicted in the genome of strain N1Y132^T by the NCBI PGAP, including 4,383 protein-coding genes, 59 tRNAs, 3 rRNAs, and 3 ncRNAs. The amino acid sequence of strain N1Y132^T are annotated by KEGG (**Supplementary Figure 3**), and 1976 genes (39.1%) could be assigned a putative function, including carbohydrate metabolism (225 genes), signaling and cellular processing (213 genes), genetic information processing (194 genes), amino acid metabolism (126 genes), environmental information processing (115 genes) and metabolism of cofactors and vitamins (107 genes), glycan biosynthesis and metabolism (78 genes) and energy metabolism (70 genes). The results of *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T and the comparison with strain N1Y132^T were shown in **Supplementary Figure 3**.

Four *Carboxylicivirga* genomes are similar in the gene number and protein function prediction. The homologous gene regions have been showed by the progressiveMauve algorithm (**Supplementary Figure 4**). Subsequently, core genes and specific genes of four strains were analyzed with a threshold of 40% pairwise identity and 0.4 length difference cutoff in amino acid (**Figure 2**). These four strains shared 2082 core genes. The genome of *C. linearis* FB218^T seem to hold the most unique genes of 1766. The resulting dDDH values between the genomes of strain N1Y132^T and *C. sediminis* JR1^T, *C. mesophila* JCM 18290^T, and *C. linearis* FB218^T are 18.5%, 18.5%, and 15.4%, respectively (**Figure 1A**). The dDDH values between strain N1Y132^T and the reference strains are less than 20%, which are far below the threshold (70%) for new species identification (Goris et al., 2007). The ANI values between strain N1Y132^T and *C. mesophila* JCM 18290^T, *C. sediminis* JR1^T, and *C. linearis* FB218^T are 74.9%, 74.8%, and 71.1%, respectively (**Figure 1A**). These values are below the proposed cut-off for a species boundary of 95-96%

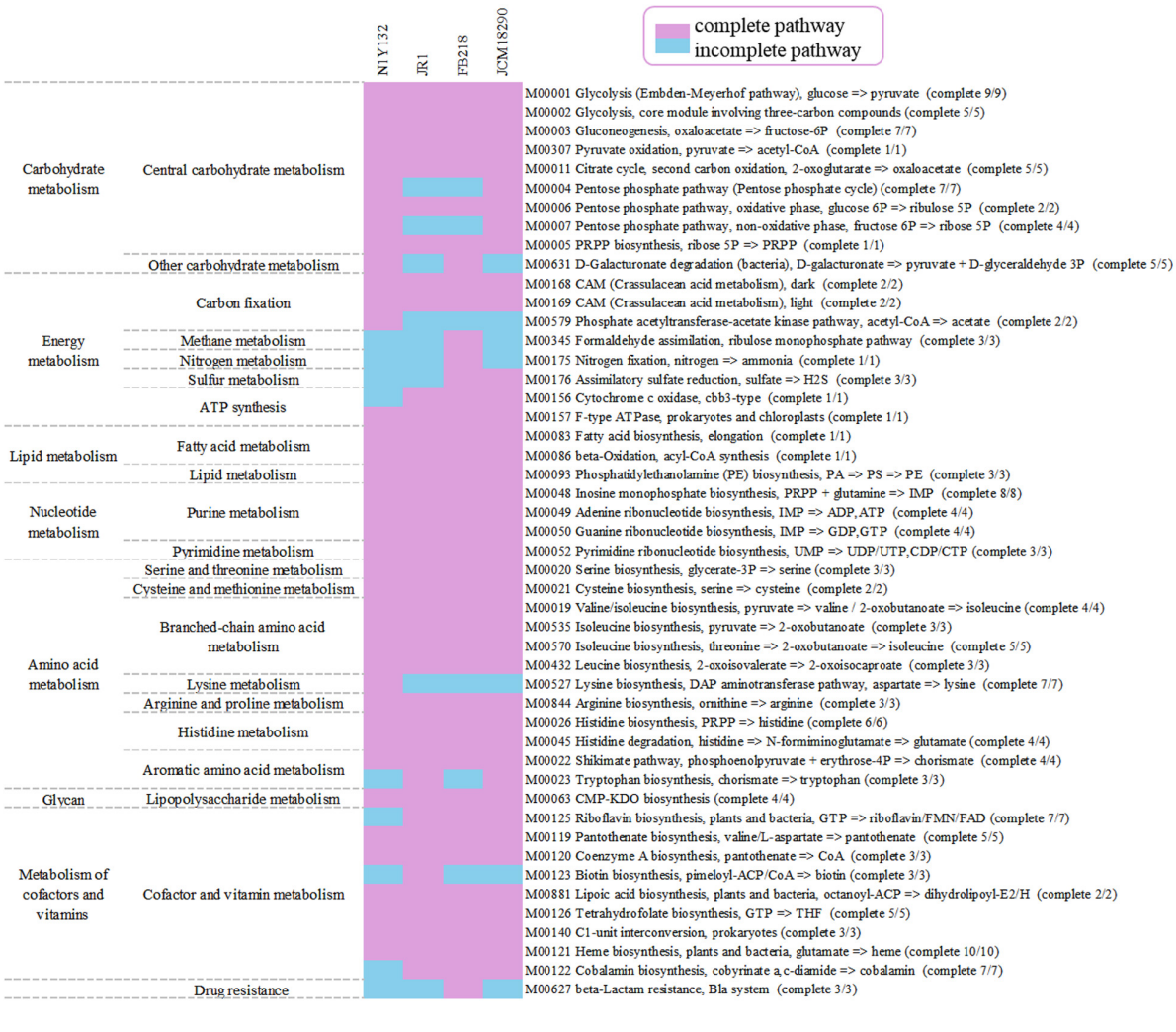

FIGURE 3 | Heat maps of complete and incomplete metabolic pathways in the genomes of strain N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T based on KEGG database.

ANI (Richter and Rossello-Mora, 2009). These results support that the new isolate represents a novel species of the genus *Carboxylicivirga*.

Metabolic Pathways

Many complete metabolic pathways have been found in the genomes of these four strains (**Figure 3**). All four strains hold the complete lipid metabolism, nucleotide metabolism and glycan metabolism. For instance, a phosphatidylethanolamine biosynthesis pathway (M00093) has been identified in these four genomes, which is in accordance with the polar lipids results of the genus *Carboxylicivirga* (**Supplementary Figure 2**). For the carbohydrate metabolism, the *Carboxylicivirga* strains possess complete pathways of glycolysis, gluconeogenesis, pyruvate oxidation, citrate cycle, pentose phosphate pathway (M00006), and phosphoribosyl diphosphate (PRPP) biosynthesis. However, the *C. sediminis* JR1^T and *C. linearis* FB218^T are deficient in the pentose phosphate pathways (M00004 and M00007), and the *C. sediminis* JR1^T and *C. mesophila* JCM 18290^T are devoid of the other carbohydrate metabolism (M00631 D-galacturonate degradation). For the amino acid metabolism pathways, only the strain N1Y132^T has the ability of lysine biosynthesis, and both *C. sediminis* JR1^T and *C. mesophila* JCM 18290^T contain the tryptophan biosynthesis pathway. For the metabolism of cofactors and vitamins, the strain N1Y132^T is lack of riboflavin, biotin and cobalamin biosynthesis, which differentiate between strain N1Y132^T and other three *Carboxylicivirga* strains.

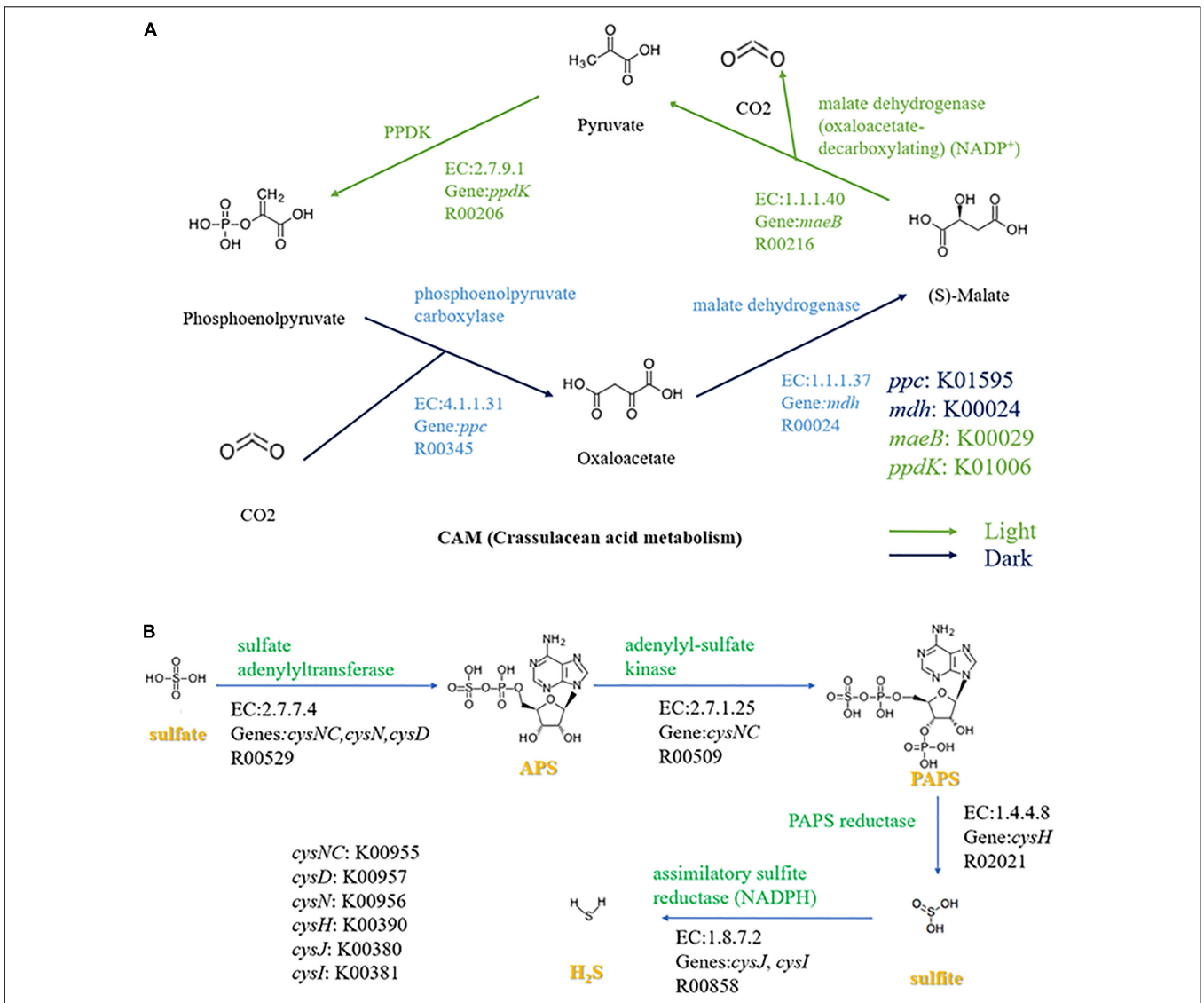

FIGURE 4 | Carbon and sulfur metabolism of *Carboxylicivirga* strains N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T. **(A)** Crassulacean acid metabolism (CAM) cycle in dark and light of four strains in genus *Carboxylicivirga*. The green arrows and blue arrows indicate the progression of light and dark reactions, respectively. **(B)** Assimilatory sulfate reduction pathway in *Carboxylicivirga* species based on KEGG analysis. Other reactants and products have been omitted.

The energy metabolism pathways exhibited the most striking difference among these four *Carboxylicivirga* strains. All strains have two carbon fixation metabolism pathways [CAM (Crassulacean acid metabolism), dark and light]. In many bacteria, CAM achieves carbon fixation through phosphoenolpyruvate carboxylase (EC: 4.1.1.31), malate dehydrogenase (EC:1.1.1.37), and PPDK (EC:2.7.9.1), the specific process is shown in **Figure 4A**. Only the strain N1Y132^T has a phosphate acetyltransferase-acetate kinase pathway, which is one of carbon fixation metabolism pathways. However, both *C. linearis* FB218^T and *C. mesophila* JCM 18290^T have a complete assimilatory sulfate reduction pathway, wherein sulfate is reduced to cysteine and sequentially incorporated into the biomass. Assimilatory sulfate reduction of *C. linearis* FB218^T and *C. mesophila* JCM 18290^T is accomplished through four steps sequentially mediated by sulfate adenylyltransferase (EC 2.7.7.4; *cysNC*, *cysN*, *cysD*), adenylyl-sulfate kinase (EC 2.7.1.25; *cysNC*), PAPS reductase (EC 1.8.4.8; *cysH*) and assimilatory sulfite reductase (NADPH) (EC 1.8.7.2; *cysJ, cysI*) (**Figure 4B**). In the genomes of the strains N1Y132^T and *C. sediminis* JR1^T, a homologous gene cluster contain *cysN*, *cysD*, *cysH*, *cysJ,* and *cysI*, but lack of the *cysNC* gene. Additionally, *C. linearis* FB218^T has a complete nitrogen fixation pathway (**Figure 3**). *C. linearis* FB218^T could grow on the mineral salts medium plates containing starch and agar but lacking NH_4^+, NO_3^-, or organic nitrogen, while other *Carboxylicivirga* strains could not grow.

Prediction of Secondary Metabolites

Based on secondary metabolite analysis predicted by antiSMASH, the genome of strain N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T and *C. mesophila* JCM 18290^T share four gene clusters encoding for the RRE (RRE-element containing cluster), the terpene cyclase, the NRPS-like (non-ribosomal peptides) and RiPP-like (Other unspecified ribosomally synthesized and post-translationally modified peptide product) cluster (**Supplementary Figure 5**). The strain N1Y132^T have 7 putative biosynthetic gene clusters (BGCs). These clusters include one RRE-containing protocluster, one RiPP-like biosynthetic cluster, two NRPS-like clusters, two terpene cyclase clusters and one hserlactone region. For bacteria living in coastal waters, the possession of multiple BGCs that can produce secondary metabolites may help them grow better (Lim et al., 2020). Besides, strain N1Y132^T and *C. linearis* FB218^T are found to have unique gene cluster, strain N1Y132^T has a hserlactone region (Homoserine lactone cluster) and *C. linearis* FB218^T has Type I PKS (Polyketide synthase).

Carbohydrate-Active Enzymes (CAZymes)

Given the polysaccharides utilization of marine *Bacteroidetes* bacteria (Krüger et al., 2019), these four *Carboxylicivirga* genomes have been analyzed by the CAZy database. Strain N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T and *C. mesophila* JCM 18290^T contain 255, 296, 333 and 287 carbohydrate-active enzymes, respectively (**Figure 5**). Among these carbohydrate-active enzymes, glycoside hydrolases (GHs) are the greatest number of enzymes (more than 60% of the identified enzymes were assigned to the GH family in the genomes of four strains). Compared with three reference strains, strain N1Y132^T harbor 255 carbohydrate-active enzymes including 172 GHs, which were obviously lower than *C. sediminis* JR1^T, *C. linearis* FB218^T and

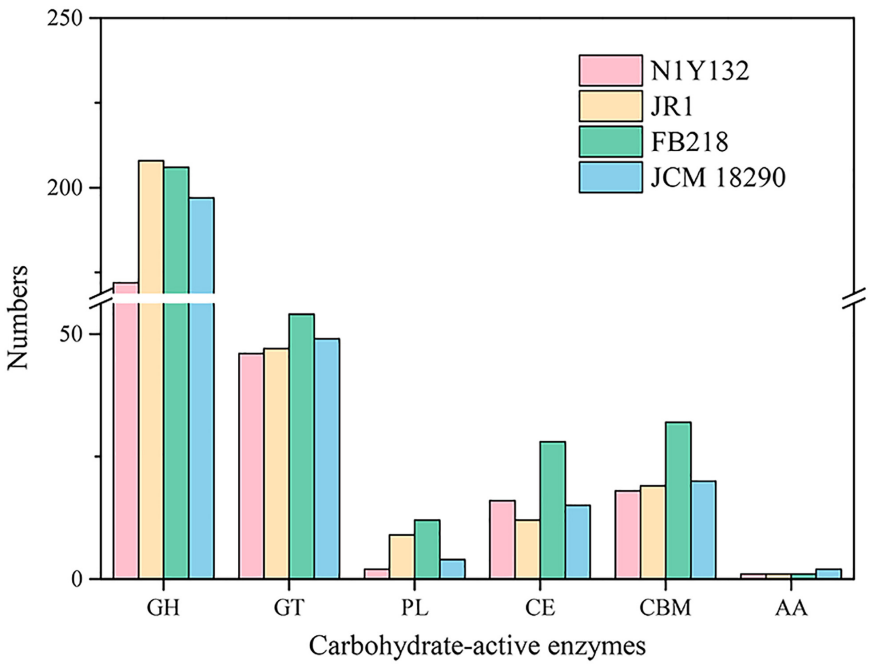

FIGURE 5 | Histogram of predicted carbohydrate-active enzymes (CAZymes) in strain N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T. Numbers of each enzyme detected in the genome were shown in the map.

C. mesophila JCM 18290^T. The results obtained from the CAZy database suggest that strain N1Y132^T and three reference strains may have a metabolic potential for polysaccharide utilization. The experimental results show that strains N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T and *C. mesophila* JCM 18290^T can utilize various carbon sources (**Table 1** and **Supplementary Table 1**), which are consistent with the genome annotation results.

According to the polyphasic taxonomic characterization results, strain N1Y132^T could be classified as a new member of the genus *Carboxylicivirga*, for which the name *Carboxylicivirga marinus* sp. nov. was proposed.

Description of *Carboxylicivirga marinus* sp. nov.

Carboxylicivirga marinus (ma.ri'nus. L. masc. adj. *marinus* of or belonging to the sea, marine).

Cells of strain N1Y132^T are slender, Gram-stain-negative, facultatively aerobic, non-motile and approximately 0.3–0.4 μm width and 5–50 μm length. Colonies on MA are circular, beige and smooth, and approximately 1.0 mm in diameter after 3 days of incubation at 28°C. Growth occurs at 15–33°C (optimal 25–28°C), at pH 6.5–8.5 (optimal pH 7.5), in 0–9% (w/v) NaCl (optimal 2.0–2.5%). Cells are negative for oxidase and catalase activity. Positive for hydrolysis of starch. Negative for hydrolyses of agar, DNA, alginate, casein, CM-cellulose and Tweens (20, 40, 60, and 80). Activities of alkaline phosphatase, alkaline phosphatase, leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-Biphosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase. Acid is produced from D-ribose, D-xylose, D-fructose (weekly), esculin, starch, D-tagatose (weekly) and potassium 5-ketogluconate. In carbon source oxidation test, positive results are obtained for 3-methyl glucose, L-histidine, glucuronamide, α-keto-glutaric acid and L-malic acid. The major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{13:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$ 3-OH, and $C_{15:1}\omega6c$. The sole respiratory quinone is MK-7. The polar lipids consist of a phosphatidylethanolamine, two phosphoaminolipids and six unidentified lipids. The DNA G + C content of the type strain is 38.1 mol%.

The type strain, N1Y132^T (= KCTC 72934^T = MCCC 1H00431^T) was isolated from marine sediment sample obtained from the coast of Weihai, China (37°34′31.44″ N, 122°9′15″ E). The GenBank accession number for the 16S rRNA gene sequence of strain N1Y132^T is MW532703 and the draft genome data has been deposited in GenBank under the accession number JAENRR000000000.

DATA AVAILABILITY STATEMENT

The GenBank accession number for the 16S rRNA gene sequence of strain N1Y132^T is MW532703 and the whole genome shotgun project of strain N1Y132^T, *C. sediminis* JR1^T, *C. linearis* FB218^T, and *C. mesophila* JCM 18290^T have been deposited at DDBJ/ENA/GenBank under the accession number JAENRR000000000, JAGTAR000000000, JAGUCO000000000, JAGUCN000000000, respectively.

AUTHOR CONTRIBUTIONS

D-CL isolated the Strain N1Y132^T. T-SZ, Y-NZ, YG, and Z-JD performed material preparation, experimental operation, data collection, and analysis. T-SZ, YG, and Z-JD wrote the manuscript. YG and Z-JD performed project guidance and critical revision of manuscripts. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (32070002, 31900091, and 31770002), the National Science and Technology Fundamental Resources Investigation Program of China (2019FY100700), the China Postdoctoral Science Foundation (2019M652369), and the State Key Laboratory of Microbial Technology Open Projects Fund (M2019-08).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.731212/full#supplementary-material

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