**Oceanisphaera pacifica** sp. nov., isolated from the intestine of *Trichiurus japonicus*

Jian-Heng Xue¹ · Li-Fang Shi² · Bei-Ning Zhang¹ · Wen-Jie Wu¹ · Yuan Gao² · Qian Zhu² · Li-Hua Zhao²

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**Abstract**

A Gram-stain-negative, strictly aerobic, non-flagellated, oxidase- and catalase-positive, rod-shaped marine bacterium, designated strain DM8ᵀ, was isolated from the intestine of *Trichiurus japonicus* in Weihai, China. The strain optimally grew at 25–35 °C, with 1.0–4.0% (w/v) NaCl and at pH 7.0–8.0. Its colonies were circular, slightly yellow, non-transparent, smooth, and approximately 0.8–1.5 mm in diameter, after being cultured for 48 h on marine agar 2216. Based on the result of phylogenetic analysis of 16S rRNA gene sequence, strain DM8ᵀ had close relationship with *Oceanisphaera profunda* SM1222ᵀ (96.9%) and the type strain DSM 15406ᵀ of the type species *Oceanisphaera litoralis* (94.7%), respectively. Genome sequencing revealed a genome size of 3,109,059 bp and a G + C content of 46.9 mol%. It had Q-8 as the sole respiratory quinone and possessed C₁₆₀, summed features 3 (C₁₆₋₁₀ ≥/ʃc/C₁₆₋₁₀ ≥/ʃc) and summed features 8 (C₁₈₋₁₀ ≥/ʃc/C₁₈₋₁₀ ≥/ʃc) as major fatty acids. The major polar lipid profile was composed of phosphatidyglycerol and phosphatidylethanolamine. Based on the phenotypic, chemotaxonomic characterizations, phylogenetic properties and genome analysis, strain DM8ᵀ should represent a novel species of the genus *Oceanisphaera*, for which the name *Oceanisphaera pacifica* sp. nov. is proposed. The type strain is DM8ᵀ (= KCTC 82764ᵀ = MCCC 1K06133ᵀ).

**Keywords** 16S rRNA gene · Aeromonadaceae · *Oceanisphaera pacifica* · Polyphasic taxonomy

**Abbreviations**

AAI Amino Acid Identity
ANI Average Nucleotide Identity
BD Becton Dickinson
dDDH Digital DNA–DNA Hybridization
DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
GGDC Genome-to-Genome Distance Calculator
HPLC High Performance Liquid Chromatography
KCTC Korean Collection for Type Cultures
KEGG Kyoto Encyclopedia of Genes and Genomes
MA Marine agar 2216
MB Marine broth 2216
MCCC Marine Culture Collection of China
MEGA Molecular Evolutionary Genetics Analysis
MIDI Microbial Identification System
TLC Thin-Layer Chromatography

**Introduction**

The genus *Oceanisphaera* in the class *Gammaproteobacteria* was first introduced by Romanenko et al. (2003) with the type species *Oceanisphaera litoralis*, isolated from marine bottom sediments. At present, ten species have been described from different environmental habitats (https://lpsn.dsmz.de/genus/oceanisphaera). The gut microbiome of fishes conveys critical roles in development, immunity, metabolism, feeding behaviour and resistance against invasive pathogens (Garcia-Gutierrez et al. 2019; Stincone and Brandelli 2020). For researching potential effects on host fish and the adaptive traits in gut, we isolated culture-dependent bacteria from the intestine of varied fishes throughout the coast of Weihai, China. In this study, strain DM8ᵀ was isolated from the intestine of *T. japonicus* and phenotypic,
chemotaxonomic and genotypic investigations showed that the strain DM8<sup>T</sup> represents a novel species affiliated to the genus Oceanisphaera.

**Materials and methods**

**Isolation, cultivation and maintenance conditions**

The fish *Trichiurus japonicus* were sampled from the coastal Weihai in China. The gut specimens were detached from fresh fish and inoculated onto marine agar 2216 (MA, BD) after being washed, homogenised, and serially diluted in sterile marine water. The culture mediums were incubated at 30℃ for 2 days. After observing morphological and pigmentation, each representative type of colony was streaked with artificial seawater (0.02% NaHCO<sub>3</sub>, 0.07% KCl, 0.12% (0.1% yeast extract, 0.5% peptone, 2.0% agar), supplying concentration on growth was examined in following medium MA in triplicate and observed every 12 h. The effect of NaCl concentration on growth was investigated at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10% (w/v) NaCl. *O. profunda* KCTC 32510<sup>T</sup> (obtained from KCTC) and *O. litoralis* DSM 15406<sup>T</sup> (obtained from Pro. Du) were used as reference strains for phenotypic, polar lipids and fatty acid analyses in this study.

**Morphological, physiological and biochemical analysis**

The morphological, physiological and biochemical characteristics of strain DM8<sup>T</sup> were tested after incubation at 30℃ for 48 h on MA. The colonies and cell morphology were performed using light microscopy (E600; Nikon) and transmission electron microscopy (JEM-1200; JEOL) at the State Key Laboratory of Bio-Fibers and Eco-Textiles (Qingdao University, China). Gram staining was performed as previously described by Smibert and Krieg (1994). Gliding motility was examined according to the method of Bowman (2000).

The temperature range for growth was investigated at 4–45℃ (4, 10, 15, 20, 25, 28, 30, 33, 37, 40 and 45℃) on MA in triplicate and observed every 12 h. The effect of NaCl concentration on growth was examined in following medium (0.1% yeast extract, 0.5% peptone, 2.0% agar), supplying with artificial seawater (0.02% NaHCO<sub>3</sub>, 0.07% KCl, 0.12% CaCl<sub>2</sub>, 0.23% MgCl<sub>2</sub> and 0.32% MgSO<sub>4</sub>, w/v) and 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10% (w/v) NaCl at 28℃, and recorded the colony growth every 12 h. Growth at different pH was tested between pH 5.5 and 9.5 (in increments of 0.5 pH units) by measuring the optical density (wavelength 600 nm) after 48 h of incubation at 30℃ in marine broth 2216 (MB, BD) with the addition of appropriate buffers, including MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.5), HEPES (pH 7.5 and 8.0), Tricine buffer (pH 8.5) and CAPSO (pH 9.0 and 9.5) at concentrations of 20 mM. Growth under anaerobic (10% H<sub>2</sub>, 10% CO<sub>2</sub> and 80% N<sub>2</sub>) and microaerobic (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>) conditions with or without 0.1% (w/v) KNO<sub>3</sub> were determined after incubation for 2 weeks at 28℃ on MA in an anaerobic jar. The susceptibility to antibiotics was performed on MA using the disk diffusion method as previously described (Jorgensen and Turnidge 2015).

The activity of oxidase was examined using an oxidase reagent kit (bioMérieux) according to the manufacturer’s instructions. Catalase activity was detected by production of bubbles after adding a drop of 3% (v/v) H<sub>2</sub>O<sub>2</sub>. Hydrolysis of agar, starch, CM-cellulose, alginate, casein and Tweens (20, 40, 60 and 80) and reduction of nitrate were investigated on MA according to previously described methods (Tindall et al. 2007). Bacteria in vitro haemolysis test was performed as described by Neter (1956). Other physiological or biochemical tests were investigated using the API 20E, API ZYM and API 50CHB identification systems (bioMérieux) and Biolog Gen III microPlates according to the manufacturer’s instructions, with the single modification of adjusting the salinity to 3% (w/v).

**16S rRNA gene sequencing and phylogenetic analysis**

The 16S rRNA gene fragment was amplified by the universal primers 27F and 1492R and purified PCR products were ligated into the PMD18-T vector (Takara) and cloned following the manufacturer’s instructions. Sequencing was performed by BGI (Qingdao, China). The returned 16S rRNA gene sequence was submitted to GenBank database and the sequences were analysed using BLAST (https://support.illumina.com) according to the manufacturer’s instructions. Phylogenetic trees based on the 16S rRNA genes were constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods with MEGA version 7.0 (Kumar et al. 2016). The stability of the phylogenetic tree was guaranteed by bootstrap analysis based on 1000 replications (Felsenstein 1985).

**Genome sequence analysis**

The total DNA of strain DM8<sup>T</sup> was extracted using a Bacteria DNA Kit (Omega) and then the purified DNA. The draft genome of DM8<sup>T</sup> was sequenced by Beijing Novogene Bioinformatics Technology, using the Illumina Hiseq Xten platform (Illumina Inc., San Diego, USA). An Illumina shotgun library using the Illumina TruSeq Nano DNA Sample Prep Kit was reconstructed and sequenced in paired end, using the Illumina HiSeq platform. Raw sequence data were generated by Illumina base-calling software CASAVA v1.8.2 (https://support.illumina.com) according to the manufacturer’s instructions. The sequenced reads were assembled using
SOAP denovo software (Li et al. 2008). The ContEst16S was used to check the contamination of DM8$^T$ genome (Lee et al. 2017). To ensure the authenticity of 16S rRNA, the complete 16S rRNA gene sequence which was obtained from the draft genome using the RNAmer 1.2 server (http://www.cbs.dtu.dk/services/RNAmer/) was compared with the PCR amplification. The G+C content of the chromosomal DNA was calculated using genome sequence. Then, multiple methods were used to annotate the gene of strain DM8$^T$. The gene of strain DM8$^T$ was determined by NCBI Prokaryotic Genome Annotation Pipeline sever online (Angiuoli et al. 2008) and the encoding genes were predicted using GeneMarkS version 4.17 (Besemer et al. 2001). The CRISPR-associated genes were annotated using CRISPRdigger version 1.0 (Grissa et al. 2007) to analyse the functional differences of different strains, especially in environmental adaptability and pathogenicity. Protein-encoding regions were identified with the Rapid Annotations using Subsysyte Technology server (Aziz et al. 2008). To identify the function of genes, a whole-genome Blast search (E-value less than 1e-5, minimal alignment length percentage larger than 40%) was performed using Gene Ontology (GO) database (Ashburner et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2015) and Cluster of Orthologous Groups of proteins (COG) database (Galperin et al. 2015). Enzymes that catalyse carbohydrate degradation, modification, and biosynthesis were predicted using Carbohydrate-Active enZYmes (CAZy) Database (Cantarel et al. 2009). The secondary metabolism gene clusters by the antiSMASH (Medema et al. 2011) was performed to predict secondary metabolites. Resistance gene prediction was achieved by Comprehensive Antibiotic Research Database (CARD) database annotation with Resistance Gene Identifier (RGI) (Jia et al. 2017) and Antibiotic Resistance Genes Database (ARDB) (Liu and Pop 2009).

To determine exact taxonomic status, a phylogenetic tree was constructed based on the core genome of the strain DM8$^T$ and its related strains. On the basis of amino acid sequences, the phylogenetic tree was analysed through FastTree (Price et al. 2010) with JTT + CAT parameters and IQ Tree (Trifinopoulos et al. 2016) with the LG + F + I + G4 model and 1000 bootstrap replicates. To analyse the genome relatedness between strain DM8$^T$ and related species of the genus Oceanisphaera, the ANI values (including OrthoANIu and ANIb values) was calculated according to online ANI calculator (https://www.ezbiocloud.net/tools/ani and http://jspecies.ribohost.com/jspeciesws) (Yoon et al. 2017b; Richter et al. 2016), the average amino acid identity (AAI) was calculated on an online AAI calculator (http://enveomics.cc.gatech.edu/aaai), and digital DNA–DNA hybridization (dDDH) was calculated by using the GGDC (http://ggdc.dsmz.de).

**Chemotaxonomic characterisation**

For the extraction of fatty acids, polar lipids, and respiratory quinones, the late exponential growth phase cultures of strain DM8$^T$ and two related strains were harvested in MB at 30 ºC for 3 days and subjected to freeze-drying. Analysis of cellular fatty acids was performed using the Sherlock Microbial Identification System (Sherlock version 4.5; database: TSBA40; MIDI) (Sasser 1990). Polar lipids were extracted and separated by two-dimensional silica gel TLC according to the procedures of Xu et al. (2007) and were analysed as method described by Minnikin et al. (1984). The respiratory quinone were extracted and analysed by HPLC according the method described by Kroppenstedt (1982).

**Results and discussion**

**Phylogenetic analysis**

The nearly full-length 16S rRNA gene sequence (1448 bp) of strain DM8$^T$ was obtained and the comparison revealed strain DM8$^T$ to be closely related to the type strain of Oceanisphaera profunda SM1222$^T$ (96.9%), followed by Oceanisphaera avium AMac2203$^T$ (96.7%). The similarity between DM8$^T$ and the type strain DSM 15406$^T$ of the type species Oceanisphaera litoralis was 94.7%. The results of the phylogenetic analysis based on 16S rRNA gene sequence showed that strain DM8$^T$ was placed within the branch constituted by the species of the genus Oceanisphaera based on maximum-likelihood method (Fig. 1), which was confirmed by the topology of trees generated by neighbour-joining and maximum-parsimony algorithms (Fig. S1, Fig. S2). In addition, a phylogenetic tree based on the genomic sequences with IQtree showed the strain DM8$^T$ formed a distinct lineage within the genus Oceanisphaera, which supported that DM8$^T$ could be assigned a novel species (Fig. 2).

**Genomic characteristics**

The result of whole-genome sequencing showed that the total length of genome was 3,109,059 bp with 37 contigs and the DNA G+C content was 46.9 mol%. The N50 length was 197,365 bp, the longest contig was 297,660 bp and the shortest was 222 bp. An average 150× coverage depth was accomplished. A total of 2,869 genes were predicted with 2740 protein-coding genes and 103 encode RNAs (6 5S rRNA, 3 16S rRNA, 7 23S rRNA, 83 tRNA and 4 ncRNA). 22 CRISPR-associated genes were annotated with 4848 bp total length. The genomic information of strain DM8$^T$
and other type strains in genus *Oceanisphaera* is listed in (Table 1).

According to the KEGG and RAST analysis, a complete phosphatidate cytidylyltransferase (EC 2.7.7.41) pathway was annotated and thus phosphatidylethanolamine (PE) can be produced in accord with the result of polar lipids experiment. Strain DM8T had the ability of biosynthesis of threonine, serine, cysteine, methionine, valine, leucine, isoleucine and histidine. For metabolism of cofactors and vitamins, pathways of NAD biosynthesis (EC 6.3.1.5), lipoic acid biosynthesis (EC 2.8.1.8) and biotin biosynthesis (EC 2.8.1.6) were found in strain DM8T genome which may provide essential vitamins for fish hosts. A complete pathway including cysNC, cysN, cysD, cysNC, cysC, cysH, cysJ and cysI genes associated with assimilatory sulphate reduction have been found in strain DM8T, *O. profunda* KCTC 32510T and *O. litoralis* DSM 15406T, which shows that the strains had the ability to absorb sulphate, convert it into sulfite (Fig. S3). However, the experiment showed that sodium thiosulfate could not be reduced to H2S. Through the existence of nitrate reductase (napA, napB), nitrite reductase (nirK), nitric oxide reductase subunit B (norB) and nitric oxide reductase subunit C (norC) indicated that nitrate could be reduced into nitrous oxide but could not produce nitrogen because the absence of nitrous-oxide reductase (nosZ) and the absence of nitrite reductase (NADH) large subunit (nirB) and nitrite reductase (NADH) small subunit (nirD) meant strain DM8T could not reduce nitrite to ammonia. However the pathway of dissimilatory nitrate reduction was completed in strain *O. litoralis* DSM 15406T which predicted its ability to produce ammonia. Gene katE (encoding catalase) and katE-intracellular protease were found in strain DM8T genome which verified its capacity to decompose hydrogen peroxide. Gene annotation results of GO and COG databases were presented in Figure S4 and Figure S5. In addition, strain DM8T genome had rsmA gene with 91.1% identity and CRP gene with 86.8% identity which mediated antibiotic efflux.
According to the data obtained, the OrthoANIu values between strain DM8T and \textit{O. profunda} KCTC 32510T was 76.4\%, below the recommended cut-off value of 95–96\% (Richter and Rosselló-Móra 2009). The AAI values between strain DM8T and \textit{O. profunda} KCTC 32510T and \textit{O. litoralis} DSM 15406T were 80.1\% and 77.8\%, below the proposed cut-off for a species boundary of 85–90\% and exceed the threshold value for a genus boundary 55–60\% (Qin et al. 2014). The dDDH values between strain DM8T and \textit{O. profunda} KCTC 32510T and \textit{O. litoralis} DSM 15406T were 22.1\% and 19.0\%, below the boundary (70\%) (Goris et al. 2007; Meier-Kolthoff et al. 2013) for new species.
identification. Moreover, the OrthoANIu values, ANIb values and dDDH values between each pair of strains in genus *Oceanisphaera* were showed in Table S1.

**Morphological, physiological, and biochemical characteristics**

The cells of strain DM8^T^ were observed to be Gram-stain-negative rods without flagella (Fig. S6). The strain DM8^T^ was found to assimilate esculin, ferric citrate, L-arabinose, D-xylose, D-turanose, potassium gluconate and D-serine, which can be distinguished from other related type strains. The strain DM8^T^ were not hydrolysed cellulose, starch, Tweens 40, 60, 80 and alginate and negative for nitrate reduction. Negative characteristics of the isolate in API ZYM, API 20E, API 50CH and Biolog Gen III microPlates tests are given in Supplementary Table S2. Further comparative analysis information between the proposed new organism and its closely phylogenetic relatives *O. profunda* KCTC 32510^T^ and *O.litoralis* DSM 15406^T^ are shown in (Table 2).

Strain DM8^T^ was found to be susceptible to (μg per disc) chloramphenicol (30), tobramycin (10), gentamicin (10), clarithromycin (15), cefotaxime (30), ceftriaxone (30), intermediate to erythromycin (15), and resistant to penicillin (10), streptomycin (10), neomycin (30), lincomycin (2), polymyxin B (300), ampicillin (10), carbenicillin (100), vancomycin (30), tetracycline (30), kanamycin

| Characteristic | 1 | 2 | 3 |
|---------------|---|---|---|
| Colony colour | Slightly yellow | Slightly yellow | Slightly greyish yellow |
| Motility | – | + | + |
| Temperature for growth (°C) | 4–35 | 4–42 | 4–35 |
| NaCl for growth (% ,w/v) | 0.5–5.0 | 0.5–10.0 | 0.5–8.0 |
| Hydrolysis of: | | | |
| Citrate | – | w | – |
| Urea | + | + | – |
| Tween 40 | – | + | – |
| Enzyme activities: | | | |
| Alkaline phosphatase | + | + | w |
| Esterase (C4) | – | – | + |
| Valine arylamidase | + | + | – |
| Acid production: | | | |
| D-ribose | w | + | + |
| L-xylose | + | – | w |
| D-fructose | + | + | – |
| D-lyxose | + | – | + |
| D-tagatose | + | w | w |
| Carbon utilisation: | | | |
| D-sorbitol | + | – | – |
| Methyl pyruvate | – | + | + |
| L-arginine | – | + | – |
| D-glucuronic acid | + | + | – |
| L-fucose | – | – | + |
| D-malic acid | – | + | + |
| D-aspartic acid | + | – | – |
| L-aspartic acid | – | + | + |
| L-serine | – | + | + |
| D-serine | + | w | + |
| DNA G+C content (mol%) | 46.9 | 58.5 | 49.8 |

All strains were rod-shaped, aerobic, oxidase- and catalase-positive and had activities for leucine aminopeptidase and acid phosphatase.

Strains: 1. DM8^T^; 2. *O. litoralis* DSM 15406^T^; 3. *O. profunda* KCTC 32510^T^. All data are from this study unless otherwise indicated. + Positive,–negative, w weakly positive.
ofloxacin (5), norfloxacin (10), rifampicin (5), which shown multiple drug resistance.

Chemotaxonomic characterisation

The major fatty acids (>10%) were C_{16:0}, summed features 3 (C_{16:1\omega7c} and/or C_{16:1\omega6c}) and summed features 8 (C_{18:1\omega7c} and/or C_{18:1\omega6c}). The summed feature 3 was the most abundant cellular fatty acid with ratio of 42.5%, which was also the most abundant fatty acid of *O. littoralis* DSM 15406^T and *O. profunda* KCTC 32510^T in ratio of 36.8% and 36.4%. The strain DM8^T can be also distinguished from other type strains in the compounds of C_{16:0} with ratio of 27.9% which was richer than other type strains. The fatty acids profiles of strain DM8^T and reference strains were shown in Table S3. The main polar lipids consist of phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and four unidentified lipids (L1–L4) (Fig. S7) which were similar to other type strains comprising the PG and PE as major polar lipids. The only respiratory quinone detected in the strain was Q-8, which was same as other related type strains.

Adaptive and host-associated features

The strain DM8^T was observed without haemolysis on the Columbia CNA Blood Agar plate after cultivation 5 days at 28°C. According to the draft genome of strain DM8^T, the strain underpinned the metabolism of amino acids, cofactors and vitamins, providing beneficial effects to the hosts (Nayak et al. 2010; Semova et al. 2012). Based on the CAZy database, carbohydrate esterases (CEs) genes (5), glycoside hydrolases (GHs) genes (20), glycosyl transferases (GTs) genes (34) and auxiliary activities (AAs) genes (3) were annotated, including β-mannosidase in GH5-18 family which verified its capacity of hydrolysis carbohydrate (Lapebie et al. 2019). The strain DM8^T was found have activity of acid phosphatase, alkaline phosphatase, urease, hydrolyse Tween 20, assisting host digestion (Wang et al. 2018). Besides, multiple drug resistance was observed based on the susceptibility test. The strain DM8^T could biosynthesise ectoine to adapt to variable osmotic pressure (Jebbar et al. 1997) according to the antiSMASH annotation. It is worth noting that DM8^T genomic function prediction shows it ability to absorb sulphate and convert it into sulfitie which seems ubiquitous in the strains of genus *Oceanisphaera*. These features may adapt strain DM8^T to tolerate to challenging environment (Matias et al. 2005; Baym et al. 2016). However, it is known little regarding how gut microbiota affect the host trophic level and metabolic activity.

Description of *Oceanisphaera pacifica* sp. nov

*Oceanisphaera pacifica* (pa.ci’fi.ca. L. fem. adj. pacificus peaceful, pertaining to the Pacific Ocean).

Cells are Gram-stain-negative, aerobic, slightly yellow, rod-shaped, non-spore-forming, non-flagellated, non-enzymatic oxidase- and catalase-positive, approximately 0.5–0.9 µm wide and 1.0–4.0 µm long. After incubation at 30°C on the MA for 48 h, the colonies are round, smooth and slightly yellow. Growth occurs between 4 and 35°C (optimum 25–35°C), pH 6.5–8.5 (optimum pH 7.0–8.0) and in the presence of 0.5–5% (w/v) NaCl (optimum 1–4%). Growth does not occur under anaerobic conditions on MA or on MA supplemented with 0.1% (w/v) KNO₃. Positive for Tween 20 hydrolyase, urease, alkaline phosphatase, valine arylamidase, naphthol-AS-Bl-phosphohydrolase, d-ribose, d-tagatose, potassium 5-ketogluconate, d-sorbitol, l-alanine, d-glucuronic acid, d-aspatic acid, acetic acid, stachyose and fusidic acid. The sole respiratory quinone is Q-8. The major cellular fatty acids are summed features 3 (C_{16:1\omega7c}/C_{16:1\omega6c}) (42.5%), C_{16:0} (27.9%) and summed features 8 (C_{18:1\omega7c}/C_{18:1\omega6c}) (11.8%). The major polar lipids are phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content of the type strain is 46.9 mol%.

The type strain, DM8^T (= KCTC 82764^T = MCCC 1K06133^T), was isolated from a hairtail intestinal in Weihai, China.

The GenBank accession number for the 16S rRNA gene sequence of *Oceanisphaera pacifica* DM8^T is MW725306 and the draft genome has been deposited in GenBank under the accession number JAGDFX000000000.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-02928-4.

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Author contributions JHX and LFS: isolated the strain DM8^T, analysed most of the data. JHX, BNZ, WJW and YG: performed the experiments. The initial draft of the paper was written by JHX, LHZ and QZ: conceived of the study, designed the study, critically revised the manuscript and co-corresponding the study. All authors read, discussed the results and revised the manuscript.

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Declarations

Conflict of interest Authors declare that there is no conflict of interest.
Ethical approval In this study, experiments were not performed on live vertebrates. Instead, freshly caught dead fish was used and this article does not contain any study with human participants or animal experiments by any of the authors.

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