Aquisphaera insulae sp. nov., a new member in the family Isosphaeraceae, isolated from the floating island of Loktak lake and emended description of the genus Aquisphaera

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Abstract Strain JC669T was isolated from a floating island of Loktak lake, Manipur, India and shares the highest 16S rRNA gene sequence identity with Aquisphaera giovannonii OJF2T. The novel strain is aerobic, Gram negative, light pink-coloured, non-motile, NaCl intolerant and spherical to oval-shaped. It grows in the form of single cells or aggregates and possibly forms structures which appear like fruiting bodies. Strain JC669T grows well up to pH 9.0. The isolate produces MK-6 as respiratory quinone, C_{18:1}^{9c}, C_{16:0} and C_{18:0} as major fatty acids and phosphatidylcholine, an unidentified amino lipid, an unidentified choline lipid (UCL) and six additional unidentified lipids (UL1, 2, 3, 4, 5, 6) as polar lipids. Strain JC669T has a large genome size of 10.04 Mb and the genomic G + C content was 68.5 mol%. The genome contained all genes essential for lycopene related carotenoid biosynthesis. The polyphasic analysis of its phylogenetic position, morphological, physiological and genomic features supports the classification of strain JC669T as a novel species of the genus Aquisphaera, for which we propose the name Aquisphaera insulae sp. nov. Strain JC669T (= KCTC 72672T = NBRC 114306T) is the type strain of the novel species.

Keywords Planctomycetes · Isosphaeraceae · Aquisphaera · Fruiting body · Sporotan

Abbreviations
NCBI National Centre for Biotechnology Information
gANI Genome average nucleotide identity
AAI Average amino acid identity
dDDH Digital DNA–DNA hybridization
HPLC High performance liquid chromatography
KCTC Korean collection for type cultures
NBRC Biological Resource Centre
PGAP Prokaryotic genome annotation pipeline
Introduction

The phylum *Planctomycetes* belongs to the PVC superphylum (Wagner and Horn 2006; Wiegand et al. 2020) and its members are ubiquitously distributed (Rensink et al. 2020; Wiegand et al. 2020). Characterized strains belonging to the phylum were mostly isolated from brackish and marine sources (Bondoso et al. 2014, 2015; Yoon et al. 2014; Lage et al. 2017; Wiegand et al. 2018, 2020; Kumar et al. 2020a,b; Peeters et al. 2020). Planctomycetes were also suggested to be untapped producers of small bioactive molecules with potential antimicrobial properties (Grac¸a et al. 2016; Jeske et al. 2015). The analysis of planctomycetal genomes yielded up to 15 biosynthetic gene clusters (BGCs) with a potential link to the production of small bioactive molecules (Wiegand et al. 2020).

Among the different families in the class *Planctomycetia* of the phylum *Planctomycetes*, the family *Isosphaeraceae* is currently comprised of the six genera *Paludisphaera*, *Aquisphaera*, *Singulisphaera*, *Tundrisphaera*, *Isosphaera* and *Tautonia*. Except for *Singulisphaera* and *Tautonia*, all the other genera of this family currently only hold a single species (Kaushik et al. 2020). The hitherto characterized members of the family *Isosphaeraceae* are mesophilic, non-motile and their cells divide by polar budding. The genus *Aquisphaera* was first described by Bondoso et al. (2011). Currently, its sole member is *Aquisphaera giovanonii*, for which the respective type strain OJF2T was isolated from sediments of a freshwater aquarium. The genus *Aquisphaera* is characterized by diphosphatidylglycerol and phosphatidylcholine as major polar lipids and C16:0 and C18:1\textsubscript{x9c} as major fatty acids. The type strain of *A. giovanonii* is strictly aerobic, having doubling time of 1–2 days, chemoheterotrophic, spherical, pink-pigmented and reproduces through budding (Bondoso et al. 2011).

The chosen sampling location in this study, Loktak lake, has an area of about 289 km\textsuperscript{2} and is the largest freshwater lake of Northeast India. Loktak lake is famous for its floating islands (Phumdis), which are heterogeneous masses of vegetation, soil and organic matter at various decomposition stages (Reddy et al. 2005). Phumdis constitute a dense rhizosphere extending down to the sediment of the lake and hence serve as an ecological habitat for several groups of bacteria, e.g. *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Planctomycetes* (Puranik et al. 2016). The lake is an ecological hotspot with a remarkable diversity of flora and fauna and was declared as Ramsar site (a wetland site designated to be of international importance) in 1990. Based on the results of a previous metagenome study (Puranik et al. 2016), strain JC669\textsuperscript{T} was isolated from the rhizosphere soil of a plant from the floating island of Loktak lake as part of a sampling and isolation campaign targeting planctomycetes (Kumar et al. 2020a, b, c, 2021a, b; Kaushik et al. 2020). In this study, we report for the first time an axenic culture of a planctomycetes strain (JC669\textsuperscript{T}) isolated form Loktak lake which has a 16S rRNA gene sequence identity (96.8\%) close to *A. giovanonii*. We characterized strain JC669\textsuperscript{T} using a polyphasic approach and compared its morphological, physiological, and genomic features to the current closest relative *A. giovanonii* OJF2\textsuperscript{T}, the type species of the genus *Aquisphaera*.

Materials and methods

Isolation, cultivation and preservation

Rhizospheric soil of *Zizania latifolia* (commonly known as Manchurian wild rice) of the phumdis (floating island) of the phumdis (floating island) was collected from Loktak lake located in the Northeast state of Manipur, India (exact location: 24°30’21”N 93°47’43”E). At the time of sample collection, the sampling spot had a pH of 7.0 and temperature of 18°C. The rhizospheric soil samples were subjected to enrichment and cultivation in a medium (Kaushik et al. 2020) containing (g l\textsuperscript{-1} in distilled water; pH 7.0); N-acetylglucosamine, 2.0; KH₂PO₄, 0.1; peptone, 0.1; yeast extract, 0.1; vitamin solution, 10 ml l\textsuperscript{-1}; Hutner’s basal salts, 20 ml l\textsuperscript{-1} - prepared in distilled water and antibiotics (streptomycin, 0.4; ampicillin, 0.2; cycloheximide, 0.025). Vitamin solution contained (mg l\textsuperscript{-1}); vitamin B₁₂, 0.2; biotin, 4; thiamine-HCl, 2H₂O, 10; Ca-pantothenate,
10; folic acid, 4.0; riboflavin, 10; nicotinamide, 10.0; p-aminobenzoic acid, 10; pyridoxine HCl, 20. Hunter’s basal salts contained (g l⁻¹); nitrilotriacetic acid, 10; MgSO₄·7H₂O, 30; CaCl₂·2H₂O, 3.5; (NH₄)₆MoO₇·4H₂O, 0.01; FeSO₄·7H₂O, 0.1; and metal stock solution 50 ml. Metal stock solution contain (g l⁻¹); Na-EDTA, 0.25; ZnSO₄·7H₂O, 1.1; FeSO₄·7H₂O, 0.018. The rhizospheric soil sample (100 mg) was mixed with 10 ml medium in a 20 ml transparent serum vial sealed with a butyl rubber stopper and incubated at 25°C. After five months of incubation, light pinkish biomass appeared at the bottom of the serum vial. This biomass was streaked on solid medium and further cultivated. The light pink colonies were maintained on the agar plates by repeated streaking and were preserved at 4°C. The obtained strain was designated JC669ᵀ.

Isolation of genomic DNA and 16S rRNA gene sequence analysis

The axenic culture of strain JC669ᵀ was used to isolate genomic DNA using the Nucleo-pore gDNA Fungal Bacterial Mini Kit (M/s. Genetix Biotech Asia Pvt. Ltd, India). The 16S rRNA gene was amplified by PCR using the planctomycetes-specific primer F40 (Köhler et al. 2008) and the universal primer R1388 (Stackebrandt et al. 1993). The amplified 16S rRNA gene was sequenced by Agri Genome Pvt. Ltd. (Kochi, India).

Genomic and in silico analyses

Genome sequencing was outsourced to AgriGenome Pvt. Ltd, Kochi, India. The Illumina HiseqX10 platform was used for whole genome sequencing (WGS) and a sequence coverage of 100 x was generated using the paired-end library. The tool Unicycler was used for de novo assembly (Wick et al. 2017) with default k-mer sizes and for further downstream analysis, Unicycler Assembly was used. Contamination of the genome sequence was checked using the ContEst tool of EZBiocloud (https://www.ezbiocloud.net/tools/contest16s; Yoon et al. 2017) which showed a single 16S rRNA gene sequence of only one organism. The genome of A. giovannonii OJF2ᵀ with the accession number NZ_CP042997 was further used for all genome-based analyses.

Annotation of the genome was performed using NCBI–PGAP online freely available RAST server (http://rast.theseed.org/Fig/rast.cgi) (Aziz et al. 2008). EzBioCloud (https://www.ezbiocloud.net/tools/ani) (Yoon et al. 2017), Genome-to-Genome Distance Calculator (GGDC 2.1) (http://ggdc.dsmz.de/distcalc2.php) (Auch et al. 2010) and AAI calculator developed by the Konstantinidis lab (Rodriguez and Konstantinidis 2014) were used for estimating OrthoANI, dDDH and amino acid identity (AAI) scores, respectively between strains JC669ᵀ and A. giovannonii. The values of percentage of conserved proteins (POCP) between two strains was calculated as described by Qin et al. (2014). Additionally, in silico metabolic pathway annotation was performed using KEGG (Kyoto Encyclopedia of Genes and Genomes) mapper, hosted by Kanehisa Laboratories and antiSMASH5.1 (http://antismash.secondarymetabolites.org) (Blin et al. 2019) was used for carrying out the in silico metabolic characterization including identification of biosynthetic gene clusters (BGCs) related to small bioactive compound biosynthesis in strain JC669ᵀ and A. giovannonii. For both strains, functional annotation including Clusters of Orthologous Groups of proteins (COG) was performed using eggnog-mapper web service (http://eggnog-mapper.embl.de) and carbohydrate active enzymes (CAZy) were determined using the dbcan meta server (http://bcb.unl.edu/dbCAN2/) by choosing default parameters.

Phylogenetic analysis

The ContEst16S service of EZBiocloud was used to extract the full length 16S rRNA gene sequence (1514 nt) from the genome of strain JC669ᵀ. The full length 16S rRNA gene sequences of all type species of the family Isosphaeraceae were obtained from EZBioCloud. The full length rpoB gene sequences encoding the beta subunit of RNA polymerase were extracted from genomes of all the type species belonging to the family Isosphaeraceae together with strain JC669ᵀ was used for phylogenetic tree construction. 92 core genes were extracted from publicly available genomes of Isosphaeraceae family members using the Up-to-date Bacterial Core Gene (UBCG) tool (Na et al. 2018). The sequences were aligned using MUSCLE
implemented in MEGA7.0 (Kumar et al. 2016). The distances were calculated by using the Kimura 2 parameter (Kimura 1980) and pair-wise deletion was applied for missing/gaps data treatment. For construction of the phylogenetic tree based on 16S rRNA gene sequences, rpoB gene sequences and 92 concatenated core gene sequences, MEGA7 was used using minimum evolution (ME), Neighbor-joining (NJ), and maximum likelihood (ML) methods (Kumar et al. 2021a, b) with 1000 replications (Felsenstein 1985).

Physiological analysis

Ten ml of basal medium (Bondoso et al. 2011) was used to test the organic substrate and nitrogen source utilization pattern of strain JC669T and A. giovannonii DSM 22561T (= OJF2T) with slight modifications. Yeast extract (0.05% w/v) was supplemented to the basal medium and 0.1% w/v (NH₄)₂SO₄ was used as nitrogen source. For the analysis of carbon substrate utilization, various carbon substrates (0.1% w/v) were tested for both strains. Similarly, for nitrogen source utilization, glucose (0.1% w/v) was used as a carbon source and different nitrogen sources (0.1% w/v) were tested for their utilization as described earlier by Kaushik et al. (2020). Vitamin B₁₂ requirement and nitrate reduction (Smibert et al. 1981) of the strain JC669T and A. giovannonii DSM 22561T were tested in liquid basal media as described by Kaushik et al. (2020). Basal medium containing 0.1% w/v glucose and 0.1% w/v (NH₄)₂SO₄ as carbon and nitrogen sources, respectively was used to test NaCl and temperature tolerance of strain JC669T and A. giovannonii DSM 22561T. The API ZYM kit (Biomerieux, France) was used to assay enzymatic activities of strain JC669T and A. giovannonii following the manufacturer’s protocol.

Chemotaxonomic characterization

For the analysis of polar lipids and fatty acids of the strain JC669T and A. giovannonii DSM 22561T (= OJF2T) cells were grown in 1.5 L medium as described above at 25 °C, pH 7.5 with yeast extract (0.05% w/v), 0.1% w/v (NH₄)₂SO₄ and glucose (0.1% w/v). Analysis of fatty acids was performed from cells harvested by centrifugation (8000 g for 15 min at 4 °C) when the cell density reached 70% of the maximum optical density (100% = OD₆₆₀ of 0.9). The methylated cellular fatty acids were analysed from the data generated by M/s Royal Research Labs, Secunderabad, India. The analysis was performed according to the Microbial Identification System’s instructions (Microbial ID; MIDI 6.0 version; RTSBA6; Sasser, 1990). Polar lipids of strain JC669T and A. giovannonii DSM 22561T were extracted, separated and characterized as previously described by Kates (1972) and Oren et al. (1996). Quinones were extracted and analyzed using HPLC analysis as described by Imhoff (1984). Polyamines were identified using HPLC following an extraction protocol which was previously described (Kumar et al. 2020b).

“Sporotan” staining of structures resembling fruiting bodies

The protocol used by Senthilnathan et al. (2020) and Ali et al. (2020) was adapted in this study. Late stationary phase liquid cultures of strain JC669T and A. giovannonii DSM 22561T (obtained after a cultivation time of approximately 4 months) were pelleted (8,000 rpm at 4 °C for 5 min), washed and resuspended in PBS buffer (pH 7.8). 100 μl of the pellet was incubated with “sporotan” (6 μl of 4 mM “sporotan” solution in DMSO) at room temperature (~ 26 °C) for 20 min and fluorescence was analyzed using a confocal laser microscope (Carl Zeiss LSM900).

Microscopy

A Carl Zeiss LSM880 microscope was used for observing cell shape, morphology, size and cell division. For scanning electron microscopic (SEM) studies, two millilitres of culture was centrifuged at 6000 g for 15 min at 25 °C. The resulted cell pellet was washed by suspending in phosphate buffer (0.05 mM, pH 7.2) and centrifuged at 6000 g for 10 min at 25 °C. The pellet was re-suspended in 0.25% (v/v) glutaraldehyde solution and kept for overnight incubation at 4 °C. Dehydration of cells were done by washing the cell pellet sub sequentially with increased concentration of ethanol from 10 to 100% (v/v) (10% interval) and finally the cells were resuspended in 100% ethanol. The sample was kept on small size coverslips kept on the SEM stab with help of adhesive tape. Finally, SEM stabs were kept for gold...
sputtering and then viewed under a SEM (Philips XL30). For the transmission electron microscopic (TEM) studies, sample preparation was similar to SEM. The ultrathin sectioning of the bacterial isolates were outsourced to CCMB, Hyderabad and sections of bacteria mounted on copper grids were viewed under a H-7500 Hitachi microscope.

Results and discussion

Phylogenetic inference

A nucleotide BLAST of the 16S rRNA gene sequence (1514 nt) of strain JC669T yielded the highest sequence identity of 96.8% to *A. giovannonii*. This value is below the species threshold of 98.7%, but above the genus threshold of 94.5%, indicating that strain JC669T is a member of the genus *Aquisphaera*, but not of the species *A. giovannonii*. Values of percentage of conserved proteins (POCP) and average amino acid identity (AAI) between strain JC669T and *A. giovannonii* turned out to be 67.3% and 76.6%, respectively. These values are above the respective threshold for delineation of genera (Luo et al. 2014; Qin et al. 2014) and thus provide further support that strain JC669T belongs to the genus *Aquisphaera*. OrthoANI and dDDH values between JC669T and *A. giovannonii* are 81.4% and 24.3%, respectively, which are below the recommended cut-off values of 95–96% for ANI and 70% for dDDH for species delineation (Chun et al. 2018; Meier-Kolthoff et al. 2014). Taken together, all analyzed phylogenetic markers suggest that strain JC669T represents a novel species of the genus *Aquisphaera*. The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1), *rpoB* gene sequences (Fig S1) and a multilocus sequence analysis tree based on comparison of 92 core genes (Fig. 2) are in accordance with the distinct clustering of strain JC669T with *A. giovannonii*.

Genomic characteristics

Based on the NCBI–PGAP analysis, the genome size of strain JC669T is 10.04 Mb with an N50 value of 90.612; the genome size of *A. giovannonii* OJF2T is 10.37 Mb. The genome of strain JC669T harbours 7451 genes (of which 7294 are protein-coding), 3 16S rRNAs, 94 tRNA genes and 77 pseudogenes. The genome of *A. giovannonii* harbours a total of 7815 genes (of which 7632 are protein-coding), 3 16S rRNAs, 94 tRNA genes and 77 pseudogenes. The G + C content of the genome of strain JC669T is 68.5 mol% which is about 3.2% lower than the G + C content of 70.8% in *A. giovannonii*. The difference in the G + C content between the strains indicates that these two are distinct species when taking into account that a 3% difference in the G + C content was recommended for species delineation (Ritcher and Rossello 2009).

The PATRIC software was used to align the genome sequences and to identify the multiple maximal matches and local collinear blocks (LCBs; www.patricbrc.org) of strain JC669T and *A. giovannonii*. The results show significant differences in the alignments of the LCBs in both strains (Fig. S2), which could be due to the shuffling or inversion of genes due to DNA rearrangement or recombination of homologous regions. The majority of proteins of strain JC669T share 60–80% similarity with those of *A. giovannonii* (Fig. S3). Both strain JC669T and *A. giovannonii* share 20–30% similarity in terms of protein sequence identity with other members of the family *Isosphaeraceae* (Fig. S3).

Genome-based analysis of the metabolism

The annotated genomes show that both strain JC669T and *A. giovannonii* contain the complete Embden-Meyerhof-Parnas pathway, TCA cycle and both oxidative and non-oxidative parts of the pentose phosphate pathway (Table S1). In silico metabolic characterization by KEGG annotation shows that strain JC669T and *A. giovannonii* are able to synthesize isopentenyl pyrophosphate (IPP) via the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP) pathway (non-mevalonate pathway). These five carbon isoprene units are precursors for quinone and carotenoid biosynthesis (Eisenreich et al. 2004; Zhao et al. 2013). In the genomes of strain JC669T and *A. giovannonii*, major genes encoding for carotenoid biosynthetic enzymes like 15-cis-phytoene synthase (EC:2.5.1.32), phytoene desaturase (EC 1.3.99.26), diapolyisoprene oxygenase (EC 1.14.99.44) and zeaxanthin glucosyltransferase (EC 2.4.1.276) are present. The enzyme 15-cis-phytoene synthase (EC:2.5.1.32) catalyzes the condensation of two molecules of geranylgeranyl diphosphate to give
phytoene diphosphate, followed by rearrangement
of the cyclopropylcarbinyl intermediate to 15-
cis-phytoene, which in turn leads to formation of lycopene
as a product. The study conducted by Kallscheuer et al.
(2019) shows that pink to red colour of the colonies in
Planctomycetes is mainly due to lycopene that is
formed as an intermediary compound of MEP/DOXP
pathway and other yet to be identified carotenoids. The
annotated genome of strain JC669 T showed that it has capability for aerobic respiration
and fermentation, which is well supported by the
presence of fermentative enzymes like acetaldehyde
dehydrogenase (EC 1.2.1.10), aldehyde dehydroge-
nase (EC 1.2.1.3), lactate dehydrogenase (EC 1.1.1.27), and alcohol dehydrogenase (EC 1.1.1.1).

The predicted fermentative products could be acetic
acid, lactic acid, butanol and acetone.

The genome mining also revealed, in strain JC669 T
(but absent in A. giovan-
nonii OJF2 T), the genes nifH, nifD, and
nifK, that encode structural components of molybde-
num-dependent (Mo-dependent) nitrogenase that help in
dinitrogen fixation by converting dinitrogen to
ammonia. The genome annotation of strain JC669 T
and A. giovanonii also showed incomplete pathway of
nitrification, involving conversion of ammonia to
nitrate. The putative gene encoding for methane/
ammonia monoxygenase which helps in conversion
of ammonia to hydroxylamine, an intermediary step in
nitrification, is absent in both strains. The genome
annotation also revealed the presence of genes coding
for enzymes like alcohol dehydrogenase (NADP+)
[EC:1.1.1.2], gluconolactonase
[EC:3.1.1.17],

\begin{figure}
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic relationship of strain JC669 T, A. giovanonii OJF2 T and other species within the family Isosphaeraceae
on the basis of 16S rRNA gene sequence similarity. The phylogenetic tree was constructed with MEGA 7 and Gimesia
maris DSM 8797 T (family Planctomycetaceae) was used as
outgroup. The GenBank accession numbers for 16S rRNA gene
sequences are shown in parentheses. Bootstrap values are shown
as percentages at the nodes corresponding to NJ/ML/ME
analysis. Bar, 0.02 nucleotide substitution per position.}
\end{figure}
carboxymethylenebutenolidase [EC:3.1.1.45], mucocarboxy methylase cycloisomerase [EC:5.5.1.1] and protocatechuate 3,4-dioxygenase beta subunit [EC:1.13.11.3] which probably involve in aromatic hydrocarbon metabolism. Strain JC669\textsuperscript{T} is predicted to have genes coding for beta-Lactam resistance, Bla system pathway based solely on the putative gene annotation and the same were not observed in \textit{A. giovannonii}. Strain JC669\textsuperscript{T} showed genes coding for enzymes like Chitinase (EC 3.2.1.14), N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25) and glucosamine-6-phosphate deaminase (EC 3.5.99.6) which helps in chitin and N-acetylglucosamine metabolism. Strain JC669\textsuperscript{T} showed no genes coding for N-acetylglucosamine-6-sulfatase and N-sulfoglucosamine sulfohydrolase that help in degradation of sulfated glycopolymers.

CAZy predicted that both, strains JC669\textsuperscript{T} and \textit{A. giovannonii} have similar genes responsible for encoding carbohydrate-active enzymes (CAZymes). Both strains JC669\textsuperscript{T} and \textit{A. giovannonii} have a large number of carbohydrate binding molecules (CBMs), followed by glycosyl transferases (GTs) and glycoside hydrolases (GHs). The major families in both strains are GH0, GT4, CBM48, GT2, CBM32 and CBM35 and are likely to be arranged in clusters (Fig. S4) and also most of these enzymes in both strains are predicted to be involved in the extracellular hydrolysis of substrates as they contain N-terminal signal peptides.

Twenty two functional categories of proteins were identified using Clusters of Orthologous (COG), proteins belonging to the categories, ‘general functional prediction only’ (R) and ‘nuclear structure’ (Y) were not observed in both strains. Both strains showed a similar pattern and number of COG categories with a few minor differences, in which a majority of the proteins belong to category S (unknown function) followed by energy production and conversion (C) and transport and metabolism (Fig. S5). Both strains are predicted to show resistance against antibiotics like fluoroquinolone and tetracycline as they have genes coding for the resistance-nodulation-cell division (RND) antibiotic efflux pump. Planctomycetes are believed to produce bioactive secondary metabolites.

\textbf{Fig. 2} Multilocus sequence analysis-based phylogenomic tree of strain JC669\textsuperscript{T} and members of the family \textit{Isosphaeraceae}. \textit{Gimesia maris} DSM 8797\textsuperscript{T} was used as outgroup. The GenBank accession numbers of genome sequences are shown in parentheses. Bar, 0.05 nucleotide substitution per position.
since they have complex life style (Wiegand et al., 2020). Strain JC669\textsuperscript{T} and \textit{A. giovannonii} have putative biosynthetic genetic clusters (BGCs) for producing secondary metabolites like Type I Polyketide Synthase (T1PKS), T3PKS, Non-Ribosomal Peptide Synthetase clusters (NRPS), heterocyst-glycolipid synthase-like PKS (hgIE-KS), terpenes and indole. Putative genetic cluster of lassopeptide is exclusive for strain JC669\textsuperscript{T}. Lanthipeptide and T3PKS genetic clusters are exclusive found in the genome of \textit{A. giovannonii} (Fig. S6).

Morphological and physiological analysis

TEM images of the strain JC669\textsuperscript{T} show cytoplasm, nucleoid region, inclusion bodies, invagination of the cytoplasmic membrane and cell division by budding (Fig. 3a, b). SEM images reveal that cells of strains JC669\textsuperscript{T} (Fig. 3c, d) and \textit{A. giovannonii} (Fig. 3e, f) are spherical to oval-shaped with crateriform structures evenly distributed over the entire surface (Fig. 3c–f). Both, strain JC669\textsuperscript{T} (Fig. 3c, d) and \textit{A. giovannonii} (Fig. 3e, f) produce structures resembling fruiting bodies. Free spore-like bodies were also observed (Fig. 3d) which got stained (Fig. S7a,b) with the spore-specific fluorescent stain “sporotan” (Senthil-nathan et al., 2020; Ali et al., 2020). Out of curiosity we have analyzed the genome for genes encoding putative proteins involved in fruiting bodies/spore formation. The analysis is based on a list of proteins involved in the formation of endospores in \textit{Firmicutes}, e.g. \textit{Bacillus subtilis} and exospores in \textit{Actinobacteria}, e.g. \textit{Streptomyces coelicolor}. BLAST analysis points towards the presence of putatively important sporulation proteins in strain JC669\textsuperscript{T} and \textit{A. giovannonii}. However, their percentage of identity is low and mainly in the range of 20–40% (data not shown). Thus, we could do not draw any conclusions in this study with regard to the formation of fruiting bodies and spores among the two taxa since it requires additional experimental support which exceeds the scope of the present study. However, it points to such a possibility, requiring an in depth analysis.

NaCl tolerance over a concentration range of 0–7% w/v (with an interval of 0.5%) was tested for strain JC669\textsuperscript{T} and \textit{A. giovannonii} at 25 \textdegree C and pH 7.0. Strain JC669\textsuperscript{T} and \textit{A. giovannonii} did not grow in the presence of NaCl even at 0.5%. The range and

![Image](image-url)
optimum temperature (5 °C–45 °C, at 5 °C interval) required for cell growth was tested at pH 7.0. The temperature range allowing for growth of strain JC669^T is between 10 °C and 30 °C and optimal growth was observed between 20 and 25 °C. The optimum temperature for the cell growth of A. giovannonii is 20–30 °C. The range of pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) allowing for growth of strain JC669^T and A. giovannonii was tested at 25 °C in buffered medium as previously described by Bondoso et al. (2011) with few modifications as described earlier. The optimum pH for growth of both strains was 7.0 and growth was observed up to pH 9.0 in both strains. Strain JC669^T and A. giovannonii were unable to reduce nitrate. Vitamin B_{12} is not required for growth of strain JC669^T while strain A. giovannonii required the same for growth. Strain JC669^T utilizes α-D-glucose, lactose, sucrose, D-galactose, mannose, maltose, D-xyllose, starch, Na-propionate, inositol, rhmannose, Na-succinate and sorbitol. Fumarate, fructose, ascorbate, acetate, pyruvate, mannitol, malate, inulin, benzoate and citrate do not support growth. D-alanine, L-arginine, glycine, L-histidine, L-leucine, L-isoleucine, L-glutamine, L-methionine, DL-ornithine, D-valine, L-proline, L-phenylalanine, D-threonine, peptone, N-acetyl glucosamine, yeast extract, casamino acids and sodium nitrate are used as nitrogen sources. L-serine, L-lysine, L-glutamic acid, L-aspartic acid, L-tyrosine, cysteine, L-tryptophan, and urea do not support growth as nitrogen source. Comparative results from growth experiments with various organic substrates or nitrogen sources between strain JC669^T and A. giovannonii are provided in Table 1.

The analysis of enzymatic activities for strain JC669^T and A. giovannonii was performed using the API ZYM kit. Strain JC669^T tested positive for the activities of alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-B1-BD-phosphohydrolase. Strain JC669^T did not show acid phosphatase, lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, cysteine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, trypsin, N-acetyl-β-glucosaminidase and α-fucosidase activity. A. giovannonii tested positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), valine arylamidase, trypsin, α-chymotrypsin, leucine arylamidase, acid phosphatase, β-glucosidase, α-glucosidase, cysteine arylamidase and negative for naphthol-AS-B1-BD-phosphohydrolase, lipase (C14), β-galactosidase, α-mannosidase, α-galactosidase, β-glucuronidase and N-acetyl-β-glucosaminidase.

Chemotaxonomic characterization

The fatty acid profile of strain JC669^T was found to be similar to that of the type species of the genus. Both strains mainly contain C_{18:1}ω9c, C_{16:0} and C_{18:0} as major fatty acids. Anteiso-C_{15:0}, C_{14:0}, C_{20:0}, C_{17:1}ω8c, C_{18:1}ω7c, and summed feature 3 fatty acids are present in both strains. In contrast, iso-C_{19:0}, iso-C_{16:0}, C_{19:0}, C_{20:0}, C_{17:1}cyclo, and C_{20:1}ω9c are exclusively found in strain JC669^T. C_{15:0} and C_{17:0} turned out to be exclusive for A. giovannonii (Table S2).

The polar lipid profile of strain JC669^T (Fig. S8a) was considerably different from that of A. giovannonii (both from this study [Fig. S8b] and from the literature [Bondoso et al., 2011]). Polar lipids of strain JC669^T include phosphatidylcholine (PC), phosphatidylglycerol (PG), an unidentified amino lipid (AL), an unidentified choline lipid (UCL) and three unidentified lipids (UL1–5) (Fig. S8a). For A. giovannonii, we identified PC, PG and three unidentified lipids (UL2,4,5; Fig. S8b). Strain JC669^T differs from A. giovannonii by the presence of an unidentified amino lipid and an unidentified choline lipid. However, the major polar lipids of A. giovannonii in this study (Fig. S8b) are similar to what was reported earlier, except for the absence of unidentified glycolipids and diphosphatidylglycerol (Bondoso et al., 2011). MK-6 is the predominant quinone in both strains. Both strains have two polyamines; sym-homospermidine and putrescine, while A. giovannonii produces an additional unidentified polyamine (Fig. S9).

Conclusion

Strain JC669^T resembles the type strain of its phylogenetically closest neighbour, regarding major polar lipids and major fatty acids. However, strain JC669^T has a 3.2 mol% lower G + C content than A. giovannonii. The phenotypic differences (Table 1) support the results of the phylogenetic inference that delineate
strain JC669T from the species *A. giovannonii*, but not from the genus *Aquisphaera*. We propose to assign strain JC669T to a new species, for which we introduce the name *Aquisphaera insulae* sp. nov. Further, our study highlighted for the first time the possibility of spores as a dormant stage in the life cycle of Planctomycetes.

### Description of Aquisphaera insulae sp. nov.

(in’su.lae. L. n. insula, island; L. gen. n. insulae, from an island, referring to the isolation of the type strain from floating island of Loktak lake, Manipur, India).

Colonies are light pink in colour. Cells are Gram-stain-negative, round- to oval- shaped, non-motile and divide by budding. Stalks and flagella are not observed. Cells do not grow in presence of additional NaCl, are strictly aerobic, heterotrophic and mesophilic. The temperature optimum is at 25 °C (range 10–30 °C). Vitamin B<sub>12</sub> is not required for growth. α-

### Table 1  Differential characteristics of strain JC669<sup>T</sup> and *A. giovannonii* DSM 22561<sup>T</sup> (= OJF2<sup>T</sup>)

| Characteristics                  | Strain JC669<sup>T</sup> | *A. giovannonii* |
|----------------------------------|--------------------------|------------------|
| Cell shape                       | Spherical to oval        | Spherical        |
| Cell size (diameter in μm)       | 1.8–2.2                  | 1.6–2.0          |
| **Organic substrates used for growth** |                          |                  |
| D-xylose                         | +                        | –                |
| Inositol                         | +                        | –                |
| Na-succinate                     | +                        | –                |
| Sorbitol                         | +                        | –                |
| D-fructose                       | –                        | +                |
| D-mannitol                       | –                        | +                |
| Acetate                          | –                        | +                |
| Pyruvate                         | –                        | +                |
| Sorbitol                         | –                        | +                |
| Nitrogen sources used for growth |                          |                  |
| Na-nitrate                       | +                        | –                |
| L-phenylalanine                  | +                        | –                |
| L-serine                         | –                        | +                |
| Aspartate                        | –                        | +                |
| Cysteine                         | –                        | +                |
| Serine                           | -                        | +                |
| Vitamin B<sub>12</sub> requirement | –                      | +                |
| **Polar lipids**                 |                          |                  |
| AL                               | +                        | –                |
| UCL                              | +                        | –                |
| **Fatty acids**                  |                          |                  |
| iso-C<sub>19:0</sub>             | +                        | –                |
| iso-C<sub>16:0</sub>             | +                        | –                |
| C<sub>15:0</sub>                 | –                        | +                |
| C<sub>17:0</sub>                 | –                        | +                |
| C<sub>20:0</sub>                 | +                        | –                |
| C<sub>20:1</sub>-cyclo           | +                        | –                |
| Genomic DNA G + C content (mol %) | 68.5                    | 70.8             |

Data from this study

Both strains produce MK-6 as respiratory quinone; both strains grow from pH 7–9 with optimal growth at 7.0. Phosphatidylcholine and six unidentified lipids are common for both strains; major fatty acids are C<sub>18:1</sub>ω9c, C<sub>16:0</sub> and C<sub>18:0</sub>; glucose supports growth; does not require vitamin B<sub>12</sub> for growth and N-acetylglucosamine is not obligate for growth; PG, phosphatidylglycerol; AL, unidentified amino lipid; UCL, unidentified choline lipids. +, substrate utilized; -, substrate not utilized;
D-glucose supports good growth. Nitrate is not reduced. Major fatty acids are C_{18:1}ω9c, C_{16:0} and C_{18:0}-MK-6 is the only respiratory quinone. Phosphatidylcholine, phosphatidylglycerol, an unidentified amino lipid, unidentified choline lipid and five unidentified lipids were identified as polar lipids. Sym-homospermidine and putrescine are the major polyamines. The genome of the type strain is characterized by a size of 10.04 Mb and a G + C content of 68.5 mol%. The type strain JC669^T (= KCTC 72672^T = NBRC 114306^T) was isolated from a rhizosphere soil of *Zizania latifolia* of the floating island from Loktak lake, Manipur, India.

The GenBank/EMBL accession number of 16S rRNA gene sequence of the strain JC669^T is LR782133, the Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAALJH000000000.

Emended description of the genus *Aquisphaera*
Bondoso et al. 2011

The description of the genus *Aquisphaera* is as given previously (Bondoso et al. 2011), with the following additions/modifications: Cells harbour crateriform structures on the cell surface and can be spherical or ovoid in shape. The genome size varies from 10.0–10.4 Mb. The DNA G + C content varies from 68 to 71 mol%. Sym-homospermidine and putrescine are the major polyamines. The major polar lipids are phosphatidylcholine and phosphatidylglycerol. The genus belongs to the family *Isosphaeraceae*, order *Isosphaerales*, class *Planctomycetia*, phylum *Planctomycetes*.

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Author contributions KL performed sample collection, KG performed media optimization and polar lipid analysis, KG and KL isolated the strain, performed the initial cultivation, strain deposition and strain characterization; KG and DK performed the electron microscopic analysis; JU performed the genomic and phylogenetic analysis; SA performed and analyzed the data for polyamines; GMK and KG performed genome mining for genes related to sporulation; KG and KL wrote the manuscript; Ramana and Sasikala supervised the study and contributed to text preparation and revised the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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