Paludisphaera rhizosphaereae sp. nov., a new member of the family Isosphaeraceae,isolated from the rhizosphere soil of Erianthus ravennae

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Abstract

Two axenic cultures of *Planctomycetota* were isolated from distinct geographical locations of India. Strain JC665^T was isolated from a rhizosphere soil of Loktak lake, Manipur, whereas strain JC747 was isolated from a sediment located at Pallikkara village, Kerala, India. The two closely related strains shared the highest (94.6%) 16S rRNA gene sequence identity with *Paludidphaera borealis* PX4^T, while the 16S rRNA gene sequence identity between both strains was 100%. Both strains grow aerobically, stain Gram negative, colonies are light pink-coloured, cells are non-motile, spherical to oval-shaped and tolerate NaCl up to 2% (w/v). While strain JC665^T grows well up to pH 9.0, strain JC747 up to pH 8.0. Respiratory quinone for both strains was MK-6. $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{18:0}$ are the major fatty acids. Phosphatidylcholine, two unidentified glycolipids, seven unidentified lipids and two unidentified phospholipid was the polar lipid composition of both strains. Both strains have genome size of about 8.0Mb and have GC content of 66.4 mol%. Both strains contained genes encoding for lycopene related carotenoids. The phylogenetic position, morphological, physiological and genomic features support the classification of strain JC665^T as a new species of the genus *Paludisphaera*, for which we propose the name *Paludisphaera rhizosphaerae* sp. nov. Strain JC665^T (= KCTC 72671^T = NBRC 114305^T) and JC747 are the type and non- type strain of the new species, respectively.

Introduction

The phylum *Planctomycetota* is a member of PVC superphylum along with *Verrucomicrobiota, Chlamydiota, Lentispaerota, Kiritimatiellaeota and Candidatus Omnitrophica* superphylum (names of the phyla used are as per the changes recommended by Oren and Garrity, 2021 and notified in the validation list by Oren and Garrity, 2022). Members of the *Planctomycetota* are ubiquitous and play crucial role in the global carbon and nitrogen cycle (Kuenen, 2008; Wiegand et al. 2018) and recognised for their used in medical and in biotechnological (Rivas-Marín and Devos, 2018; Wagner and Horn, 2006). These members are frequently (70–85%) isolated from various biotic and abiotic surfaces of marine habitats, including macroalgae, sponges and sediments (Kohn et al. 2020; Wiegand et al. 2020; Gaurav et al. 2021) but is seldomly isolated from the biotic and abiotic surfaces of freshwater habitats (Kumar et al. 2021). Other than aquatic habitats, the sole use of N-acetyl glucosamine or in combination with glucose as carbon source and by exploiting the natural resistance of planctomycetal strains to several antibiotics also led to the isolation of its several axenic strains from different terrestrial habitats like soil, peatland and wetland (Kaushik et al. 2020).

Recent studies showed that India harbour rich diversity of *Planctomycetota* which is well supported by the isolation of several axenic strains from different habitats including Loktak lake (Kumar et al. 2021; Gaurav et al. 2021; Kaushik et al. 2020). Loktak 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. It has an area of about 289 km^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, including *Planctomycetota* (Puranik et al. 2016; Kumar et al. 2021)

The family *Isosphaeraceae* is currently comprised of six genera i.e *Paludisphaera, Aquisphaera, Singulisphaera, Tundrisphaera, Isosphaera* and *Tautonia.* Till date, the characterised members of the family *Isosphaeraceae* are mesophilic, non-motile, oval to pear shaped cells and divide by polar budding. The genome size ranges from 5.4–10.4 Mb while DNA GC content ranges from 62.2–71.1% (Bondoso et al.2015; Imhoff,1984; Kohler et al.2008; Kohn et al. 2020; Keunen et al.2008). The genus *Paludisphaera* was described by (Kulichevskaya et al. 2006) to accommodate a new planctomycete of the family *Isosphaeraceae* isolated from a boreal Sphagnum peatbog. Recently, another species, "*Paludisphaera soli"* was added to this genus (Kaushik et al. 2020) which was isolated from a high-altitude soil in the Western Himalaya.

In this study, we describe a new member of *Paludisphaera*, isolated from two distinct and distantly located (≈ 4000 km) ecosystem; aquatic and wetland. Strain JC665^T is isolated from the rhizosphere soil of *Erianthus ravennae* from the floating island of Loktak lake, Manipur (northeast part of India), whereas strain JC747 is isolated from the wetland ecosystem of Pallikkara village, Kerala (southwest part of India). Combining a polyphasic taxonomic approach together with genomic information, we conclude strain JC665^T as the type strain of a new species of the genus *Paludisphaera* for which we propose the name *Paludisphaera rhizosphaerae* sp. nov. and strain JC747 as its non-type strain.

Materials And Methods

Habitat and isolation

Rhizosphere soil of *Erianthus ravennae* (commonly known as "Plume grass") of the "phumdis" (floating island) was collected from Loktak lake located in the Northeast part of India, Manipur (exact location: 24°30'21" N 93°47'43" E). Sediment sample was collected from the wetland located (village: Pallikkara) in the southwest part of India, Kerala (12° 23' 02" N 75° 02' 33" E). At the time of sample collection, samples had a pH of 7.0 and temperature of 22°C. The rhizosphere soil and sediment sample were used for enrichment and cultivation in a medium (Kumar et al. 2021) containing (g l^{-1} in distilled water; pH 7.0): *N*-acetylglucosamine, 2.0; KH_2PO_4, 0.1; peptone, 0.1; yeast extract, 0.1; vitamin solution, 10 ml l^{-l}; Hutner's basal salts, 20 ml l^{-l} prepared in distilled water. The antibiotics (g l^{-1}) streptomycin, 0.4, ampicillin, 0.2 and cycloheximide, 0.025 were added to the media. Vitamin solution contained (mg l^{-1}): vitamin B_{12}, 0.2; biotin, 4; thiamine-HCl.$2H_2O$, 10; Calcium pantothenate, 10; folic acid, 4.0; riboflavin, 10; nicotinamide, 10.0; p-aminobenzoic acid, 10; pyridoxine HCl, 20. Hunter's basal salts contain (g l^{-1}): nitrilotriacetic acid, 10; $MgSO_4.7H_2O$, 30; $CaCl_2.2H_2O$, 3.5; $(NH_4)_6MoO_7O_{24}.4H_2O$, 0.01; $FeSO_4.7H_2O$, 0.1; and metals stock solution 50 ml. Metal stock solution contain (g l^{-1}): Na-EDTA, 0.25; $ZnSO_4.7H_2O$, 1.1;

$FeSO_4.7H_2O$, 0.5; $MnSO_4.H_2O$, 0.15; $CuSO_4.5H_2O$, 0.04; $Co(NO_3)_2.6H_2O$, 0.025; $Na_2B_4O_7.10H_2O$, 0.018. The samples (50 mg) were mixed with 10 ml medium in different serum vials of 50 ml capacity and the vial was sealed with butylated rubber stoppers. The serum vials were then incubated for five months at 25°C to enrich *Planctomycetes*. After five months of incubation, a light pink globular bacterial colony was observed at the bottom of the serum vial. The light pink globular colony was further streaked on an agar plate containing the same medium. After three weeks of incubation, pink colonies appeared along with white colonies on the agar plates. The pink colonies were purified through repeated streaking. Pure cultures were maintained on agar plates by repeated sub-culturing and preserved at 4ºC. Purified cultures were grown in the above medium without antibiotics, unless otherwise mentioned. The pink coloured cultures isolated from the rhizosphere soil and sediment samples were designated as strains JC665^{T}and JC747, respectively.

DNA isolation, 16S rRNA gene sequencing and BLAST analysis

Commercial DNA isolation kit (Nucleo-pore gDNA Fungal Bacterial Mini Kit, from M/s. Genetix Biotech Asia Pvt. Ltd, India) was used for the DNA isolation which was then used for 16S rRNA gene amplification and genome sequencing. For the 16S rRNA gene amplification, *Planctomycetes* specific primers F40 (Kohler et al.2008) and R1388 (Stackebrandt et al.1993) were used. For PCR amplification, TAKARA master mix (EmeraldAmp® GT PCR Master Mix) was used. Thermocycler conditions were as follows: an initial denaturation step (94 ^oC for 10 min) followed by 33 cycles of denaturation (94 ^oC for 1 min), annealing (52 ^oC for 54 s), and extension (72 ^oC for 1.4 min). Finally, the tubes were incubated at 72 ^oC for 15 min to ensure complete synthesis of the entire sequence. The amplified PCR product were sent to M/s. AgriGenomePvt. Ltd. (Kochi, India) for purification and 16S rRNA gene sequencing. Sequence was identified using BLAST search analysis on EzBioCloud database (Yoon et al.2017).

Genomic information and in-silico **metabolic characterisation**

Whole-genome sequencing (WGS) of the strains JC665^{T}and JC747 was outsourced to M/s. AgriGenome Pvt. Ltd, Kochi, India. WGS was carried out using Illumina HiseqX10 platform and paired-end libraries were generated with a sequence coverage of 100x. Unicycler assembly software (Wick et al.2017) was used for *De novo* assembly with its default k-mer sizes and it was also used for all further downstream analyses. ContEst service (Yoon et al. 2017) was used for any possible contamination. RAST server (http://rast.theseed.org/FIG/rast.cgi) (Aziz et al. 2008) was used for annotating the genomes. Average Amino acid Identity (AAI) was calculated using the AAI calculator developed by Konstantinidis lab (Rodriguez and Konstantinidis 2014). The orthologous gene clusters and distribution of proteins among the different strains were compared by constructing a heat map and a Venn diagram with the help of a web server, Orthovenn2 (https://orthovenn2.bioinfotoolkits.net/home). Carbohydrate active enzymes (CAZy) were determined using dbCAN meta server (http://bcb.unl.edu/dbCAN2/) by choosing default parameters (Zhang et al. 2018). *In-silico* metabolic characterization of the strains JC665^{T}and JC747 was carried out on the basis of its genome information by using KEGG mapper (Kanehisa, M. and Sato, Y

2020). *In-silico* identification for the presence of genetic clusters responsible for the biosynthesis of secondary metabolites was carried out using online freely available tool anti SMASH5.1 (http://antismash.secondarymetabolites.org). (Blin et al. 2019).

Phylogenetic analysis

The 16S rRNA gene sequence of the strains JC665^{T}and JC747 were extracted from its genome using ContEst16S https://www.ezbiocloud.net/tools/contest16s and analysis of identity was performed using NCBI BLAST (Johnson et al. 2008). The 16S rRNA gene sequences of strains JC665^{T}and JC747 and other members of the family *Isosphaeraceae* were aligned using the MUSCLE implemented in MEGA7.0 (Kumar et al. 2016) and the distances were calculated using Kimura 2-parameter (Kimura 1980) in a pairwise deletion procedure. Neighbor-joining (NJ), minimum evolution (ME), and maximum likelihood (ML) methods in the MEGA7 software were used to construct phylogenetic trees having bootstraps of 1000 replication (Felsenstein 1985). The phylogenomic tree was constructed using 92 core genes (retrieved using UBCG tool as described by (Na et al. 2018) from all the publicly available genomes of *Isosphaeraceae* families. A concatenated sequence of 92 genes was used to construct the RAxML based phylogenomic tree as described by Kumar et al. (2021).

Physiological analysis

For organic substrates and nitrogen source utilisation, basal medium was used as described previously (Bondoso et al. 2011) with slight modifications. Medium was supplemented with trace amount of yeast extract (0.05% w/v). For organic substrate utilisation, $(NH_4)_2SO_4$ (0.1% w/v) was used as a nitrogen source and cell growth was tested with different organic substrates at a concentration of 0.1% (w/v). For nitrogen source utilisation, glucose (0.1% w/v) was used as organic carbon source and cell growth was tested with different nitrogen substrates at 0.1% (w/v). Both organic and nitrogen substrate utilisation was tested in test tubes (25 x 250 mm) containing 10 ml of basal medium as described above. 10 ml of broth in test tubes (25 x 250 mm) was used for determining the utilization of organic carbon/nitrogen substrates and vitamin B_{12} requirements as previously described (Kaushik et al. 2020). NaCl tolerance (1–10% w/v, at an interval of 1% w/v; final NaCl concentration in the modified M30 medium) was tested at 25°C and pH 8. Optimal temperature (5, 10, 15, 20, 25, 30, 35, 40°C) required for growth was tested in modified M30 medium at pH 8. pH range (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) for growth was tested at 25°C in buffered medium as described previously by (Bondoso et al. 2015) To examine the hydrolysis of phytagel, culture was streaked onto the media solidified with 2% phytagel (Sigma-Aldrich) and incubated for 4 weeks (Kaushik et al .2020; Kulichevskaya, et al.2016). The phytagel hydrolysing property was also checked on media supplemented with *N*-acetylglucosamine as a sole source of carbon and nitrogen. Enzyme activities was assayed using API ZYM kit (Biomerieux, France) as per the manufacturer's protocol.

Chemotaxonomic characterization

For fatty acid analysis, healthy growing cells were harvested by centrifugation (10,000 *g* for 15 min at 4 ºC) at a cell density of 70% of the maximum optical density (100% = 0.9 OD660). Cellular fatty acids were

methylated, separated and identified according to instructions for the Microbial Identification System [Microbial ID; MIDI 6.0 version; method, RTSBA6 (Sasser 1990), which was carried out by Royal Research Labs, Secunderabad, India. Polar lipids were extracted, separated and characterized as described previously (Kates 1972; Oren et al. 1996). Quinones were extracted with a chloroform/methanol (2:1, v/v) mixture, purified by TLC and analysed by HPLC (Imhoff 1984). Polyamines were extracted and identified according to a recent method (Kumar et al. 2020).

Microscopy

Cell morphological features like size and shape, and cell division were observed under field emission scanning electron microscopy (FESEM) or Transmission electron microscopy (TEM). For FESEM, one ml of log-phase culture was centrifuged at 7000x*g* for 10 min at 4°C. The resulted cell pellet was washed by re-suspending in sterile MilliQ and centrifuged at 7000 *g* for 10 min at 04°C. The pellet or cells were fixed in 2.5% glutaraldehyde solution and kept for six hours incubation at 4°C. Cells were dehydrated sequentially with an increased ethanol concentration from 10–100% (v/v) (10% interval). At last, the cells were resuspended in 100% ethanol. 10µl of samples were kept on a small size glass slide, which was placed on the stab with adhesive tape (Kumar et al. 2021). Finally, stabs were kept for gold sputtering for six minutes and then cell morphology and division were viewed under the FESEM (Philips XL3O) facility of school of Physics, UOH. For TEM, ultrathin sectioning of the log phase cells was outsourced to RUSKA Diagnostic, Hyderabad. The sections of the cells were mounted on copper grids and observed under TEM (H-7500 Hitachi) facility of CCMB, Hyderabad.

Results And Discussion

Genomic characteristics, BLAST analysis and phylogenetic inference

Genome size of the strains JC665^{T}and JC747 are 8.05 Mb and 8.04 Mb with an N_{50} value of 238,467 and 226,135, respectively. Genome of the strain JC665^T has 6,431genes of which 6,307 are protein coding genes, 80 genes code for RNAs (3 genes for encoding r-RNAs, 74 genes for t-RNAs and 3 for other RNAs) and 44 genes are pseudogenes (Table S1). Genome of the strain JC747 has 6,420 genes of which 6,299 are protein coding genes, 80 genes code for RNAs (3 genes for encoding r-RNAs, 74 genes for t-RNAs and 3 for other RNAs) and 41 genes are pseudogenes (Table. S1). The organization of orthologous clusters among the strains will provide a better understanding in genome structure and gene/protein function. We could predict from the genome wide annotation, 7106, 7102, 7373 and 6643 proteins for strains JC665^T, JC747, "*P. soli*" JC670^T and *P. borealis* PX4^T respectively. The predicted proteins of strains JC665 and JC747 showed 6973, 6969 orthologous clusters and 103 singletons for which no orthologs were found in other species (Fig. S1A). "*P. soli*" JC670^T showed 4612 clusters and 2464 singletons. *P. borealis* PX4^T showed 4268 clusters and 2160 single tons (Fig S1A). The comparative analysis of orthologous gene clusters performed shows that these species formed 7499 clusters, 4032 orthologous clusters (at least

contains two species) and 3467 single-copy gene clusters (Fig. S1B). A total of 14738 proteins were present in orthologous clusters found in all the strains whereas the strain JC670 and PX4 showed 314, 190 proteins in the clusters specific to them (Fig. S1C). Further comparison of shared orthologous gene clusters showed that 3556 clusters were observed in all the strains, no unique clusters were observed in the strains JC665^T and JC747 whereas "*P. soli*" JC670^T and *P. borealis* PX4^T showed 127 and 73 unique clusters respectively (Fig. S1D).

Genomic DNA G + C content of both strains (JC665^T, JC747) is 66.4 mol% (Table S1). The 16S rRNA gene sequences of the strains JC665^{T}and JC747 extracted from the genomes have sequence length of 1521 nt. BLAST analysis of 16S rRNA gene sequence of strains JC665^T and strain JC747 in EzBioCloud server showed identity of 94.6% and 96.7% with *P. borealis* PX4^T and "*P. soli*" JC670^T, respectively (Fig. 1). Comparison of dDDH, gANI, and AAI values of strains JC665^T and JC747 with *Paludisphaera* spp. yielded similarity of (19.4–20.3) %, (62.4–68.6) %, and (75.1–77.9) %, respectively. AAI, OrthoANI and dDDH values fell well below the recommended cut-off of 80%, 95–96% and 70%, respectively for prokaryotic species delineation (Rodriguez and Konstantinidis 2014; Meier-Kolthoff et al. 2014; Chun et al. 2018). Thus, both the newly isolated strains represent a novel species of the genus *Paludisphaera*. However, high values of 16s rRNA (100%), dDDH (100%), gANI (100%), and AAI (99.9%) between strains JC665^{T}and JC747 suggest these strains to be same species from two different and distantly located ecosystems.The 16S rRNA gene sequence based phylogenetic tree with combined bootstrap values obtained from NJ, ME, ML trees (Fig. 2) and 92 core genes based phylogenomic tree (Fig. 3) confirmed the distinct monophyletic clustering of strains JC665^T and JC747 with *Paludisphaera* members within the family *Isosphaeraceae* and suggest a novel species within the genus *Paludisphaera*.

In-silico **metabolic characterisation**

In order to have better understanding on metabolic functions, the COGs annotation was performed. The results showed that both strains, JC665^T and JC747 showed similar results as that of the other members of the genus *Paludisphaera.* Most of the genes predicted were of unknown function and later followed by genes involved in energy production and conversion (Fig. S2). The CAZy annotation of genomes shows that the strains JC665^T and JC747 contains more genes encoding glycoside hydrolases followed by glycosyl transferases compared to that of other members of the genus *Paludisphaera.* The analysis also shows that 80–90% of the enzymes belonged to families of glycoside hydrolases and glycosyl transferases (Fig. S3). The presence of higher number of carbohydrate active enzymes with respect to bacterial metabolism needs further studies. *In silico* metabolic characterisation showed that strain JC665^T and JC747 have the 2-C-methyl-Derythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway for the biosynthesis of five carbon isoprene units (isopentenyl pyrophosphate), that acts as a precursor for the synthesis of carotenoids and quinones. The putative genes encoding for enzymes like 15-cis-phytoene synthase [EC:2.5.1.32], phytoene desaturase [EC:1.3.99.26 1.3.99.28 1.3.99.29 1.3.99.31] etc which helps in formation of lycopene as an end product with intermediary products like phytoene, zeta carotene and neurosporene were predicted in the genomes of both strains. The genes for assimilatory

nitrate reduction were observed only in *P. borealis* PX4^T. The putative genes for CAM (Crassulacean acid metabolism), was observed in JC665^T and JC747 only. All the strains belonging to genus *Paludisphaera* along with JC665^T and JC747 showed putative gene clusters for synthesis of Type I, Type III polyketide synthases and terpene biosynthesis. However, genes for production of indole were predicted exclusively in JC665^T and JC747 only (Fig. S4). *In-silico* genome analysis of all the four strains of *Paludisphaera* also showed the presence of the putative hopanoid biosynthesis pathway genes like squalene synthase (*hpnC)*, squalene/phytoene desaturase (*hopC*), squalene hopene cyclase (*Shc*; codes for the key enzyme of hopanoid biosynthesis), radical S-adenosyl-L-methionine (SAMe) required for addition of adenosyl group to hopane skeleton (*hpnH*), acetylornithine aminotransferase/amino-bacteriohopanetriol synthase (*hpnO*), hopanoid associated sugar epimerase (*hpnA)* and sterol desaturase family protein (*erg32*).

Morphological and physiological analysis

SEM image show that cells of strain JC665^T and JC747 are spherical to oval shaped (1.7-1.8x1.3-1.5µm; Fig. 4A) and have well distribution of crateriform structures (CR) all over the cell surface. TEM image of the cells shows the presence of cytoplasmic membrane (CM), outer membrane (OM), invagination of cytoplasmic membrane (ICM), cytoplasm (CP), nucleoid region (N), Ribosomes (RB) and cell reproduction by budding (BD) where daughter cell is protruding from mother cell (Fig. 4B).

NAG is not obligate for the growth of strains JC665^T, JC747, "*P. soli*" JC670^T and *P. borealis* DSM 28747^T. Strains JC665^T, JC747, "*P. soli*" JC670^T and *P. borealis* DSM 28747^T utilizes following organic carbon sources: α-D-glucose, sucrose, Na-pyruvate, D-galactose, mannose, rhamnose, and trehalose. Neither of the strains utilizes following organic carbon sources: starch, ascorbate, acetate, mannitol, malate, inulin, benzoate, Na-succinate and citrate. Lactose and maltose are utilized by the strains JC665^T, JC747, and *P. borealis* DSM 28747^T. Fructose and D-xylose are utilized by the strains "*P. soli"* JC670^T and *P. borealis* DSM 28747^T. Cellobiose and ribose are exclusively utilized by the strain *P. borealis* DSM 28747^T. Fumarate and propionate are exclusive for the strains JC665^T (including JC747) and "*P. soli"* JC670^T, respectively. All the strains utilizes the following nitrogen sources for their growth: ammonium sulphate, peptone, yeast extract, DL-alanine, L-arginine, casamino acid and sodium nitrate. Neither of the strains utilizes following nitrogen sources: L-aspartic acid, urea and valine. The following nitrogen sources are exclusively utilized by the strains JC665^T, JC747 and "*P. soli"* JC670^T: L-glycine, L-phenylalanine, L-lysine, L-glutamine, L-proline, L-isoleucine, L-leucine, DL-ornithine and DL-threonine. However, L-methionine and cysteine are exclusively utilized by the strains JC665^T and JC747. L-serine and L-tyrosine is exclusive for the strain "*P. soli"* JC670^T (Table 2). Strain JC665^T can hydrolyse phytagel (Fig. S5) only in the absence of N-acetylglucosamine in the medium, as also observed previously for *P. borealis* PX4 ^{T} and "*P. soli"* JC670^T (Kaushik et al.2020; Kulichevskayaet al.2016).

All the four strains showed positive for esterase (C4), leucine arylamidase, and valine arylamidase. However, all the strains show negative for lipase (C14), cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β- glucosidase, α-mannosidase, and α-fucosidase. Alkaline

phosphatase, esterase lipase (C8), and acid phosphatase are exclusively positive for the strains "*P. soli"* JC670^T and *P. borealis* DSM 28747^T. Naphthol-AS-BI-phosphohydrolase show positive for the strains JC665^T, JC747 and "*P. soli"* JC670^T. β-galactosidase and N-acetyl- β-glucosaminidase, are exclusively positive for the strain *P. borealis* DSM 28747^T only.

Chemotaxonomic Characterisation

The major fatty acids in strains JC665^T, JC747, "*P. soli"* JC670^T and *P. borealis* DSM 28747^T are $C_{18:1}\omega9c$, $C_{18:0}$ and $C_{16:0}$. In terms of fatty acids composition, significant differences were found among all the strains (Table. S2). The polar lipids of strains JC665^T and JC747 are; phosphatidylcholine (PC), two unidentified glycolipids (GL1, 2), six unidentified lipids (UL1-7) and two unidentified phospholipid (PL1, 2) (Fig. S6A). The polar lipids of strain *P. borealis* DSM 28747^T compose of phosphatidylcholine (PC), phosphatidylethanolamine (PE), one unidentified choline lipid (CL1), two unidentified glycolipids (GL1, 2), two unidentified lipids (UL1, 2), two unidentified amino lipids (AL1, 2) and four unidentified phospholipids (PL3-6) (Fig. S6B) and is not found in congruence of earlier study, as different method was adopted for the identification of polar lipids (Kulichevskaya et al. 2016). The polar lipids of strain "*P. soli"* JC670^T include phosphatidylcholine, two unidentified phospholipids and six unidentified lipids (Fig. S6C) and is found in congruence of earlier study (Kaushik et al. 2020). Polyamines of the strains JC665^T and "*P. soli"* JC670^T include sym-homospermidine and putrescine. Polyamine of the strain JC747 include spermidine and two unidentified polyamines (1, 3). Polyamine of the strain *P. borealis* DSM 28747^T include spermidine and two unidentified polyamines (2, 3) (Fig. S7). MK6 is the predominant quinone for all the strains.

Proposal of strain JC665 ^{T} as a new species of the genus Paludisphaera

Strains JC665^T and JC747 have clear phylo-genomic differences with "*P. soli"* JC670^T and *P. borealis* DSM 28747^T but between themselves they are similar species (Fig. 1,2,3; Fig. S1, Table. S1). The phylo-genomic differences are well supported by chemotaxonomic and phenotypic differences (Table. 1), which support strain JC665^T as a novel species of the genus *Paludisphaera.* For this, we propose the name of the type strain JC665^T as *Paludisphaera rhizosphaerae* and strain JC747 as its non-type strain.

Descriptions of Paludisphaera rhizosphaerae **sp. nov.**

Paludisphaera rhizosphaerae (rhi.zo.sphae'rae. Gr. n. *rhiza*, root; L. n. *sphaera*, sphere; N.L. gen. n. *rhizosphaerae*, from the rhizosphere)

Color of chemotrophically grown culture is pale pink. Cells are spherical to oval shaped, and are strictly aerobic. Cell division is through budding. NaCl is not obligate for growth and can tolerates up to 2% (w/v). Optimum pH and temperature for growth are 7.0 (range 6.0–9.0) and 25ºC (range 4–34°C) respectively. N-acetylglucosamine (NAG) is not obligate for the growth. As an organic carbon substrate, D-glucose, Sucrose, Pyruvate, D-galactose, Mannose, Rhamnose, rhamnose, inositol, fumarate, lactose, maltose,

sorbitol, and trehalose are utilized. Fructose, Na-propionate, D-xylose, starch, ascorbate, acetate, mannitol, malic acid, inulin, succinate, benzoic acid and citrate are not utilized. Ammonium sulphate, peptone, casamino acid, yeast extract sodium nitrate, L-cysteine, L-methionine, L-histidine, L-glutamic acid, L-arginine, DL- alanine, l-glycine, L-glutamine, L-proline, L-isoleucine, L-ornithine and DL-threonine are utilized as nitrogen source. L-serine, L-tyrosine, L-aspartic acid, L-tryptophan, Urea and L-valine are not utilized as nitrogen source. Hydrolyse phytagel. Major fatty acids are $C_{18:1}\omega9c$, **$C_{16:0}$**, and $C_{18:0}$. Minor fatty acids include anteiso-$C_{11:0}$, anteiso-$C_{12:0}$, $C_{13:0}$, $C_{I4:0}$, anteiso-$C_{15:0}$, $C_{15:2}OH$, $C_{15:1}\omega5c$, $C_{17:0}$, $C_{17:1}\omega8c$, anteiso-$C_{17:0}$, anteiso-$C_{17:0}$ and $C_{18:3}\omega6c,9c,12c$. Putrescine and sym- homospermidine are the major polyamines. The polar lipids phosphatidylcholine, two unidentified glycolipids (GL1, 2), seven unidentified lipids (UL1-7) and two unidentified phospholipid (PL1, 2). MK6 is the only quinone. Nitrate is not reduced. API ZYM shows positive for esterase (C4), leucine arylamidase, and valine arylamidase and Naphthol-AS-BI-phosphohydrolase. Negative for lipase (C14), cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β- glucosidase, α-mannosidase, α-fucosidase. Alkaline phosphatase, esterase lipase (C8), acid phosphatase, β-galactosidase and N-acetyl-β-glucosaminidase. The type strain JC665^T (= NBRC 114305 = KCTC 72671^T) was isolated from the rhizosphere soil of *Erianthus ravennae* (commonly known as “Plume grass”) collected from Loktak lake located in the Northeast part of India, Manipur (exact location: 24°30’21” N 93°47’43” E). JC747 is an additional strain isolated from a wetland located (village: Pallikkara) in the southwest part of India, Kerala (12° 23' 02" N 75° 02' 33" E). The GenBank accession numbers of the 16S rRNA gene sequence and genome sequence of strain JC65^T and JC747 are LR746340, OU374731 and JAALCR000000000 and JAHPZK000000000, respectively.

Declarations

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Author contributions

Khongsai L performed sample collection from Loktak lake, KG, Ramana, and Sasikala performed sample collection from Kerala, Khongsai L and KG isolated the strain, performed the initial cultivation, strain deposition and strain characterisation, KG performed media optimisation and polar lipid analysis and the electron microscopic analysis, JU performed the genomic and phylogenetic analysis, Smita N and Khongsai L performed the endometabolites and exometabolites analysis. SA and Khongsai L performed and analysed the data for polyamines, KG and Khongsai L 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.

Conflicts of interest: The authors declare that there is no conflict of interest.

Ethical statement: Not applicable

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Tables

Table. 1. Differences in the characteristics of strains JC665^T, JC747, "*P. soli*" JC670^T and *P. borealis* DSM 28747^T.

Characteristics	JC665^T	JC747	*"P. soli"* JC670^T	*P. borealis* DSM 28747^T
shape	Spherical to oval	Spherical to oval	Spherical to oval	Spherical
Cell size (LxW,in μm)	1.8-1.7x1.5-1.3	1.8-1.7x1.5-1.3	1.6-1.7x1.3-15	1.5 to 2.5
Arrangement of cell	Single or in tissue-like aggregates	Single or in tissue-like aggregates	Single or in tissue-like aggregates	Single, in pairs or short chains
pH range(optima)	6-9(7.0)	6-8(7.0)	7.0-8.0(7.0)	3.8-8.0(5.0-5.5)
NaCl range(%w/v) (Optimum)	0-2 (0)	0-2 (0)	0-2 (0)	0-3 (0)
Temperature range(optimum)	4-34(26-28)	8-30(26-28)	4-30(22-25)	4-37(15-25)
Nitrogen sources utilization				
L-Phenylalanine	+	+	+	-
L-Lysine	+	+	+	-
DL-Threonine	+	+	+	-
Glycine	+	+	+	-
L-Isoleucine	+	+	+	-
L-Glutamine	-	-	+	-
L-Proline	+	+	+	-
Carbon sources utilization				
Maltose	+	+	-	+
Succinate	-	+	-	+
Propionate	-	+	+	-
Activity of enzymes				
Alkaline phosphatase	-	+	+	-
β-Galactosidase	-	-	-	+
N-acetyl-β-glucosaminidase	-	+	-	+
Fatty acids composition				

$C_{12:0}$ anteiso	+	-	-	-
$C_{13:0}$	+	-	-	-
$C_{16:0}$ N alcohol	+	-	-	-
$C_{16:1}\omega9c$	-	-	+	+
$C_{17:0}$	+	-	+	-
Long chain [a]hydroxy fatty acid	-	-	-	+
Major Polar lipids				
Phosphatidylcholine	-	-	+	+
Phosphatidylethanolamine	-	-	-	+
Major Polyamines				
Spermidine	-	+	-	+
Sym homospermidine	+	-	+	-
Putrescine	+	-	+	
Genomic features				
G+C content(mol%)	66.4	66.4	70.4	66.3
Genome size (Mb)	8.05	8.04	7.97	7.65
Coding sequences	6351	6340	6392	5785
RNAs	80	80	61	85
CRISPRs	1	1	0	1

Data., from this study.

[a] Fatty acid with 25 or more carbon length

Figures

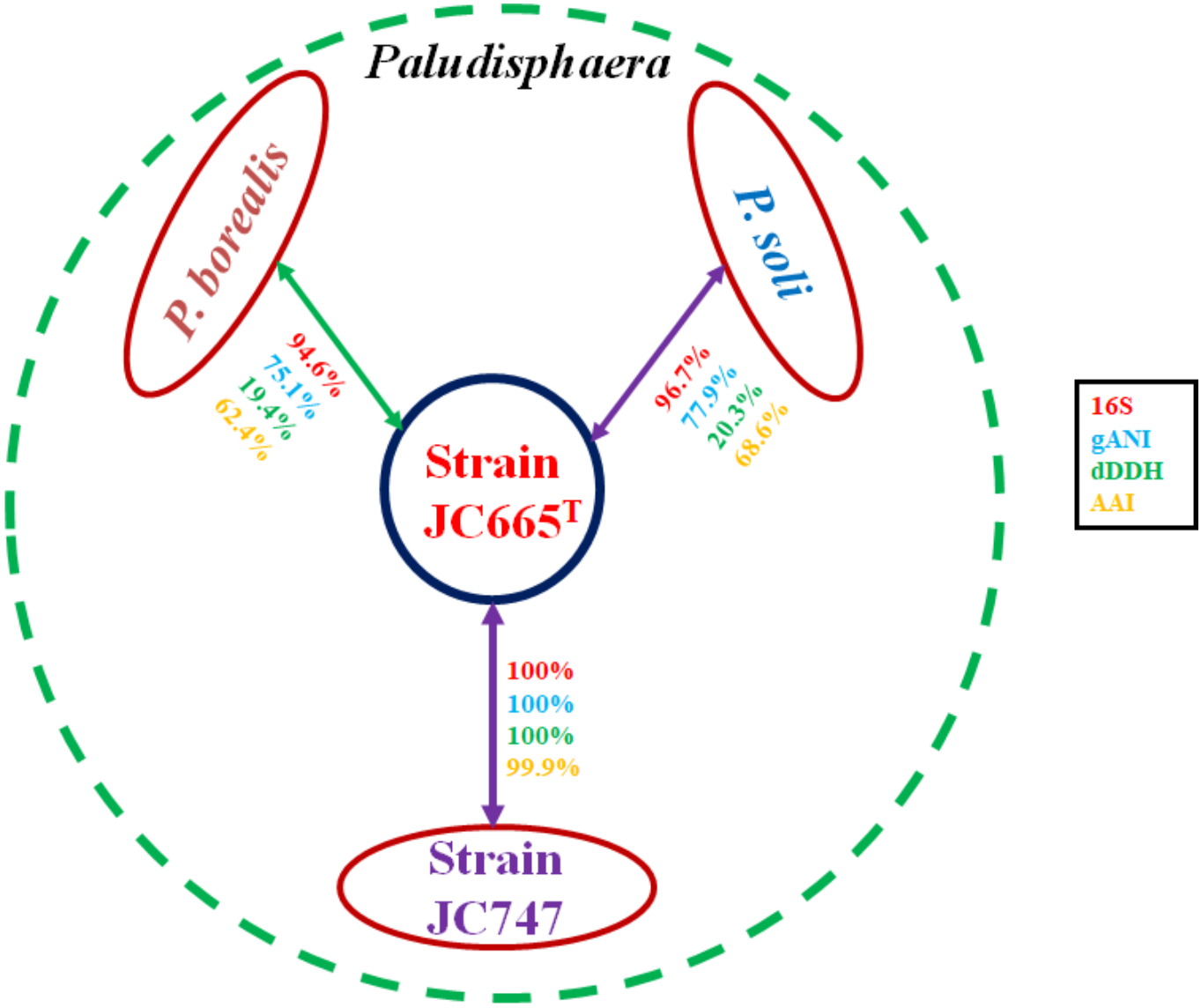

Fig. 1

Figure 1

Comparison of phylogenetic markers for delineation of the novel isolates JC665^T, JC747 with closely related species. Methods used:16S rRNA gene identity (16S), average nucleotide identity(gANI), DNA-DNA hybridization (dDDH) and average amino acid identity (AAI).

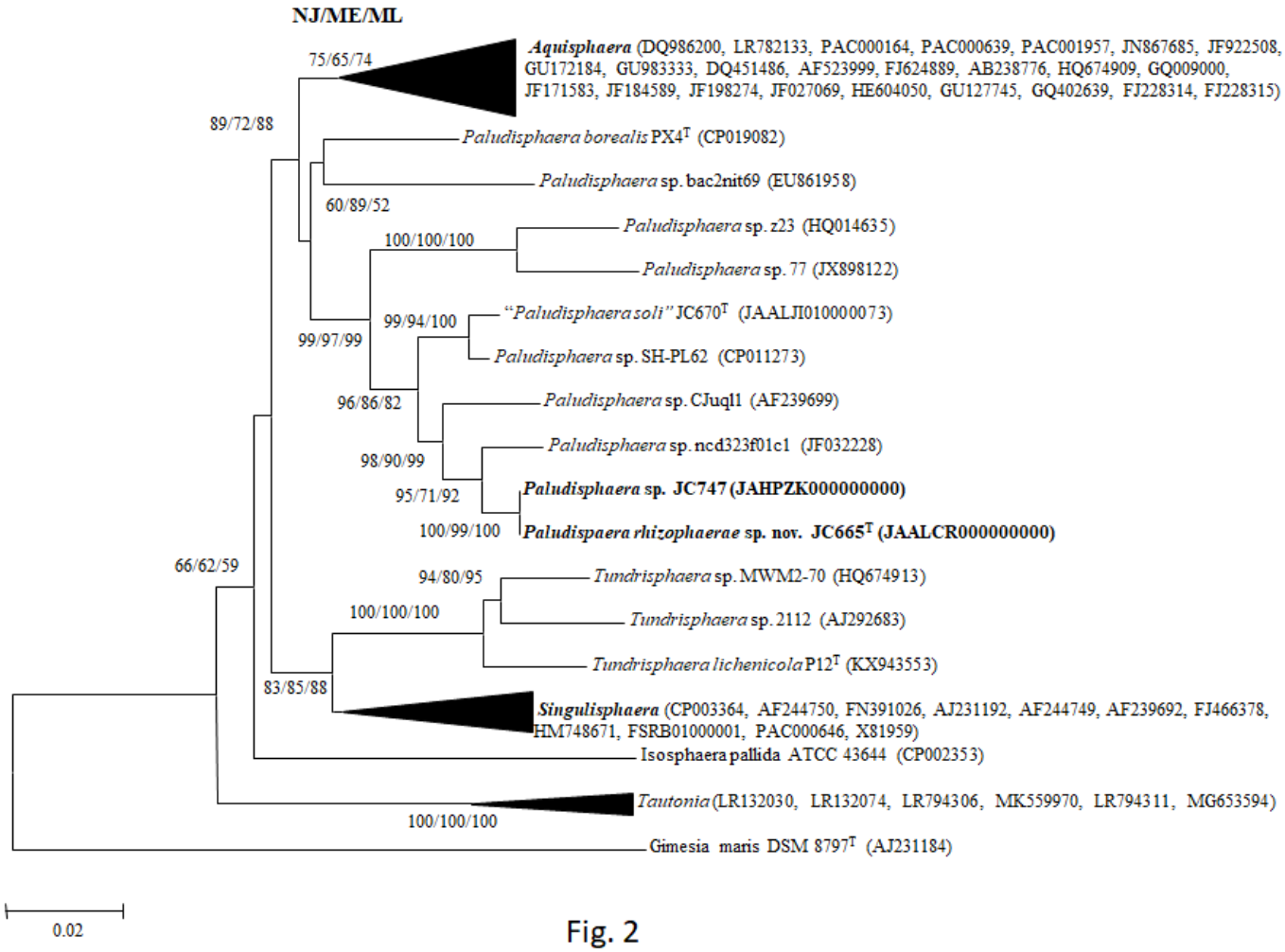

Fig. 2

Figure 2

Phylogenetic tree based on 16SrRNA gene sequences showing the phylogenetic relationship of strain JC665^{T}, JC747 and other closely related species of *Isosphaeraceae*. The tree was constructed using MEGA7 software and *Gimesia maris* DSM 8797 ^{T} was used as an out-group. The GenBank accession numbers of 16SrRNA gene sequences are shown in parentheses. Numbers at nodes indicate Bootstrap values from 1000 repetitions corresponding in the NJ/ME/ML analysis. Bar,0.02 nucleotide substitution per position.

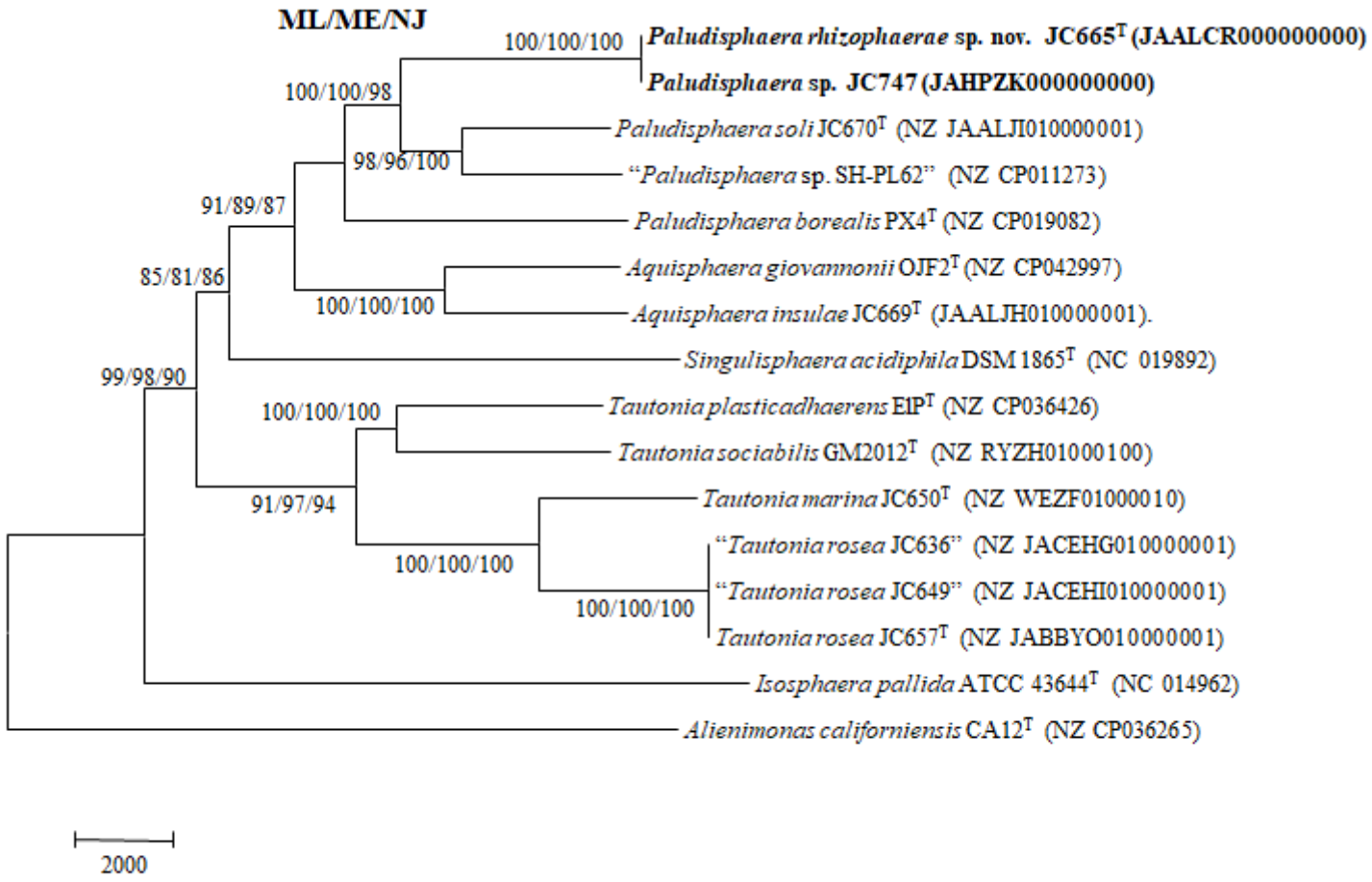

Fig. 3

Figure 3

RAxML Phylogenomic tree of strains JC665^T and JC747 along with publicly available genome sequences of *Isosphaeraceae* family. The GenBank accession numbers of genome sequences are shown in parentheses. The tree was computed with MEGA 7 software and rooted with *Alienimonas californiensis* CA12^T as the out-group. Bootstrap percentage refer to ML/ME/NJ analysis. Bar, 0.1 nucleotide substitution per position.

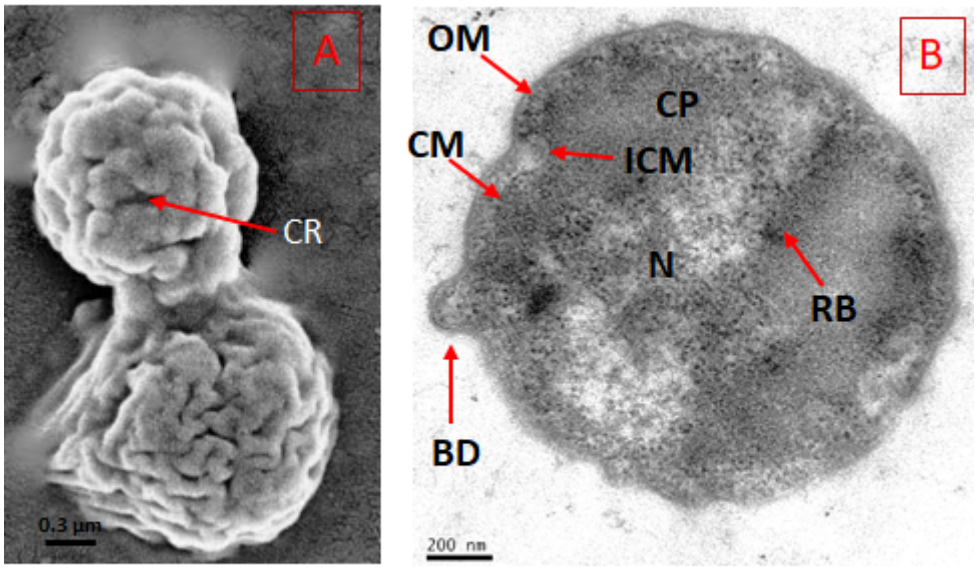

Fig. 4

Figure 4

Scanning (**A**) and transmission (**B**) electron micrographs of cell of strain JC665^T. **A.** Cells having crateriform structures (CR). Bar, 0.3 µm., **B.** Ultrathin section showing invagination of cytoplasmic membrane (ICM), outer membrane (OM), cytoplasmic membrane, cytoplasm (CP), Ribosome (RB), nucleoid region (N) and cell of strain JC665^T multiply by budding (BD) where in daughter cells are smaller than parent cells. Bar, 0.2 µm.

Supplementary Files

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