Taxonomy of the genus *Rhodobacter*

Thesis submitted for the degree of

Doctor of Philosophy

By

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CERTIFICATE

This is to certify that **Mr. SURESH G** has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. Ch. Venkata Ramana for a full period prescribed under the Ph.D. ordinances of this University. We recommend his thesis entitled **"Taxonomy of the genus *Rhodobacter*"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to declare that the work embodied in this thesis entitled **"Taxonomy of the genus *Rhodobacter*"** has been carried out by me under the supervision of Prof. Ch. Venkata Ramana, Department of Plant Sciences, School of Life Sciences. The work presented in this thesis is a bonafide research work and has not been submitted for any degree or diploma in any other University or Institute. A report on plagiarism statistics from the University Librarian is enclosed.

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This is to certify that this thesis entitled **"Taxonomy of the genus *Rhodobacter*"** is a record of bonafide work done by **Mr. Suresh G**, research scholar for Ph. D. program under the Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or institution for the award of any degree or diploma.

Parts of the thesis have been:

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1. **Suresh, G.,** Dhanesh, Kumar., Krishnaiah, A., Sasikala, Ch., and Ramana, Ch.V. (2019). *Rhodobacter sediminicola* sp. nov., isolated from a fresh water pond of Gujarat. *Int. J. Syst. Evol. Microbiol.* https://doi.org/10.1099/ijsem.0.003913.
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2	PL802	Research ethics and management	2	Pass
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Suresh G

Dedicated to my beloved parents and my supervisor

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LIST OF ABBREVIATIONS

Abbreviations	Expansion
AAI	Average amino acid identity
AAPB	Aerobic anoxygenic phototrophic bacteria
AL	Aminolipid
ANI	Average nucleotide identity
APB	Anoxygenic phototrophic bacteria
ATCC	American type culture collection
ATP	Adenosine triphosphate
BChl	Bacteriochlorophyll
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLASTP	Protein Basic Local Alignment Search Tool
bp	Base pair
BPGA	Bacterial pan-genome analysis
CDS	Coding sequence
CTP	Cytidine triphosphate
DDBJ	DNA data bank of Japan
*d*DDH	Digital DNA-DNA hybridization
DDH	DNA- DNA hybridization
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
DPG	Diphosphatidylglycerol
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
ECR	Extra chromosomal replicons
EDTA	Ethylenediaminetetraaceticacid
EMBL	European molecular biology laboratory
FAME	Fatty acid methyl esters
g	Gram
$g.l^{-1}$	Gram per liter
G+C	Guanine+cytosine
GC	gas chromatography

GC-MS	Gas chromatography mass spectrophotometry
GL	Glycolipid
GPS	Global positioning system
h	Hour
HMM	Hidden Markov Model
HPLC	High-performance liquid chromatography
HSPs	high-scoring segment pairs
ICM	Intracytoplasmic membrane
ICNB	International Code of Nomenclature of Bacteria
JCM	Japan collection of microorganisms
KCTC	Korean collection for type cultures
L	Litre
LDL	low-density lipoprotein
LH	Light harvesting
LMG	Laboratorium voor Microbiologie
lux	luminous flux per unit area
MAFFT	Multiple Alignment using Fast Fourier Transform
Mb	Mega base pairs
MBP	Modified Biebl and Pfennig's media
MCL	Markov Cluster
ME	Minimum Evolutionary
MEGA	Molecular Evolutionary Genetics Analysis
Mg	Milligram
Min	Minute
MK	Menaquinone
ml	Milliliter
ML	Maximum Likelihood
MLSA	Multilocus sequence analysis
mM	Millimolar
MUMs	maximally unique matches
NaCl	Sodium chloride
NBRC	NITE Biological Resource Center
NCBI	National Center for Biotechnology Information

NJ	Neighbor-Joining
nm	Nanometer
nt	Nucleotide
OD	Optical density
OrthoANI	Orthologous average nucleotide identity
PCR	Polymerase chain reaction
PDA	Photodiode array
PGA	Pan-genome analyses
PGAP	Pan-genome analysis pipeline
PGC	Photosynthetic gene cluster
PNSB	Purple non sulfur bacteria
POCP	Percentage of conserved proteins
PSB	Purple sulfur bacteria
pufL	Photosynthetic reaction center L subunit
pufM	Photosynthetic reaction center M subunit
pufX	Intrinsic membrane protein pufX
Q	Ubiquinone
RAST	Rapid Annotation using Subsystem Technology
Rba.	*Rhodobacter*
RC	Reaction center
Rdv.	*Rhodovulum*
RNA	Ribonucleic acid
Rps.	*Rhodopseudomas*
RQ	Rhodoquinone
rRNA	Ribosomal ribonucleic acid
s	Seconds
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TEAP	Triethylamine phosphate
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
UBCG	Up-to-date bacterial core gene

UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WGS	Whole Genome Sequencing
ΔTm	melting temperature
µg	Microgram
$\mu g l^{-1}$	Microgram per liter
(w/v)	Weight/volume
≤	Less than or equal to
°C	Degree Celsius
µl	Microliter
µM	Micromolar
$^{0}C.min^{-1}$	Degree Celsius per minute
10K	10000
2D	Two dimensional
1D	One dimensional

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“The sure and definite determination (of each species of bacteria) require so much time, so much acumen of eye and judgment, so much perseverance and patience that there is hardly anything so difficult”

— Otto F. Müller

Introduction

1. INTRODUCTION

1.1 Taxonomy

Taxonomy is a highly dynamic branch of science and requires taxon's genomic, physiological, biochemical and morphological data for classification and identification. The bacterial taxonomy consists of three main components

1. Classification; Grouping of organisms on the basis of similarity
2. Nomenclature; Naming of the groups according to the bacterial code (ICNB) (Sneath, 1992)
3. Identification; Determination of a newly isolated strain whether it belongs to an existing taxon or a new taxon

Taxonomy of a taxon may change based on the new findings, which may lead to change in the classification, nomenclature and identification procedure. Based on the classification if the strain is new it will be described as a new taxon or if the strain is already an identified one, then the new characters, if any may be added to the previous description.

1.2 Background

Antonie van Leeuwenhoek described the tiny microorganisms and named them as animalcules or little eels (later noted as prokaryotes) by viewing through his primitive microscope. Initial attempts at classification and naming of these organisms were not made until late eighteenth century (Dobell, 1932).

Bacterial classification was first proposed by Ferdinand Cohn in 1872 based on some of their morphological characters. Later in 1983, few more morphological characters were added by the bacteriologists (Palleroni, 1983). Since, the morphological differences appeared as self-evident, scientists are still using for classification. Winogradsky and Beijerinck revealed the important physiological

groups of bacteria, therefore advancing the knowledge of biochemical diversity in the microbial world considerably. Further, Mayr (1968) has commented the importance of physiological differences in bacterial classification. The ‘‘phylogenetic’’ classification of bacteria first proposed by Kluyver and van Niel in 1936, was based on the degree of morphological complexity. However, it took nearly a half century for molecular based phylogenetic classification to commence (Woese, 1987).

Edwards and Ewing (1962) and Ewing (1986) understood the disadvantage of classification and identification based on just the single character which would be insufficient, even though if that is an important character. They were expressed results in percentages by performing the large number of biochemical tests and by using more and diverse samples.

In 1950s, Numerical taxonomy came into picture and increased the validity of phenotypic identification by comparing the more phenotypic characters (100-200) of several strains (Sneath, 2015). Degree of similarity among the strains and species was calculated as matrices and resulting dendrograms revealed a general picture of the phenotypic traits.

1.3 Polyphasic taxonomy

Colwell (1970) coined the term ‘Polyphasic taxonomy’ around 50 years back, which assimilate many levels of genetic, phenotypic and chemotaxonomic information to delineate taxa. The first recommendation in the polyphasic taxonomy for species delineation is less than 70% DNA-DNA hybridization similarity (DDH) values, along with more than 5 °C difference in melting temperature **(**Δ*T*m). Phenotypic and chemotaxonomic characters should also agree with genotypic information. The delineation of higher taxa like genus and family do not have a clear definition and this has led to the creation of genera in which the genotypic and

phenotypic divergence varies based on the individual concepts of taxonomists. A polyphasic approach is commonly used for bacterial classification and analysis of evolutionary relationships by adding phenotypic, genomic and chemotaxonomic concepts, few of which are discussed below.

1.3.1 Genotypic data

In this method data derived from the DNA, RNA and proteins are used. Methods like DNA-DNA hybridization and 16S rRNA gene sequence analysis are considered as gold standard in bacterial taxonomy.

1.3.1.1 16S rRNA gene analysis

In 1970s, the sequencing of DNA and RNA were introduced and based on the studies it was concluded that, conserved region sequence comparison would give the accurate relationship between organisms. Therefore, 16S rRNA gene came into picture, which is universally present having conserved and variable regions, which allowed the usage of universal primers to study the gene across the domain bacteria and archaea. The higher taxonomic ranks like domains and phylums arised due to incorporation of 16S rRNA gene into taxonomy (Woese, 1987). This has revolutionized the prokaryotic taxonomy enabling the possibility of determining relationship between the two organisms. By comparing the 16S rRNA gene sequence and DNA-DNA re-association values, Stackebrandt and Goebel in 1994 recommended, 97% of 16S rRNA gene sequence identity for species delineation. Studies suggested that 16S rRNA gene sequence identities of 97% corresponded to not more than 60% re-association values and therefore DNA-DNA hybridization (DDH) studies would not be required in such cases. This 97% cut off was used as a gold standard for many years bur later it was shifted to 98.7% by comparing 16S

rRNA gene sequence similarities and DNA–DNA reassociation values (Stackebrandt and Ebers, 2006). The identity cut-off of 94.5% for genus, 86.5% for family, 75.0% for phylum was recommended based on 16S rRNA gene analysis of both cultured and uncultured bacteria (Rosselló-Móra and Amann, 2015; Yarza et al., 2014). However, it was proven that the cutoff value of 97.0 or 98.7% is not suitable for many genera (Beye et al., 2017, Rossi-Tamisier et al., 2015). Even though 16S rRNA gene served as good phylogenetic marker in higher ranks of taxonomy, it has poor resolution at species level which can be overcome with the simultaneous phylogenetic analysis of house-keeping called the multilocus sequence analysis (MLSA).

1.3.1.2 Multilocus sequence analysis (MLSA)

Greater phylogenetic resolution of species can be achieved by analysis of house-keeping genes like *rpoB* because,

i. House-keeping genes are less conserved compared to 16S rRNA gene

ii. Unlike 16S rRNA gene, most house-keeping genes have single copy and

iii. Rate of horizontal gene transfer is low

However, the single gene based phylogenetic analysis often leads to wrong evolutionary relationships because such trees reflect evolution of only one particular gene. In order to overcome this problem; eight or more protein coding gene sequences would be concatenated and used for phylogenetic analysis (Gevers et al., 2005; Glaeser and Kämpfer, 2015) to achieve the proper phylogenies. Based on 16S rRNA gene sequence identities, the newly isolated taxa can be identified up to the level of genus and MLSA would show further phylogenetic resolution between the species. Schleifer (2009) and Cole et al. (2010) opined that MLSA can be used as an alternate tool to DDH. Based on analysis of five house-keeping

genes, Vanlaere et al. (2009) and Vandamme and Peeters (2014) recommended 3% difference in concatenated sequences for species delineation of *Burkholderia cepacia* complex and difference was equivalent to 70% similarity in DDH. In *Salinivibrio* and *Bacillus cereus* groups 97-97.5% concatenated sequence similarity cut off was established for species differentiation (Liu et al., 2015; López-Hermoso et al., 2017). Since MLSA analysis is based on few genes, the whole genome based tools such as Average nucleotide identity (ANI) came into picture which was stated as an extension to MLSA analysis by Vanlaere et al. (2009) and Peeters et al. (2013).

1.3.1.3 DNA-DNA hybridization (DDH)

DDH technique is based on the denaturation and renaturation capability of DNA which is used to calculate the genomic relatedness between two genomes. In 1961, using DNA reassociation principle; Schildkraut et al. (1961) determined DNA-DNA relatedness of organisms interns of taxonomy. They have observed that denatured DNA of one organism will re-nature with another organism only if the both organisms are genetically related. DDH served as a gold standard in species delineation for more than 50 years. Wayne et al. (1987) recommended the 70% DDH value for species delineation, which is still followed. Even though DDH experiments have significantly served taxonomists in delineating species, it has become unpopular because of the following reasons:

i. DDH values calculated from different methods gives different values and with the same method to get reproducible value is difficult

ii. The protocol is laborious and tedious

iii. No possibility to generate the cumulative database to share among taxonomists. For every new combination of organisms, DDH has to be performed

Due to these drawbacks, taxonomists suggested replacement with more accurate and reproducible tools such as multilocus sequence analysis (MLSA), average nucleotide identity (ANI) and digital DNA-DNA hybridization. The workflow of genome based polyphasic taxonomy is given in Fig. 1.1.

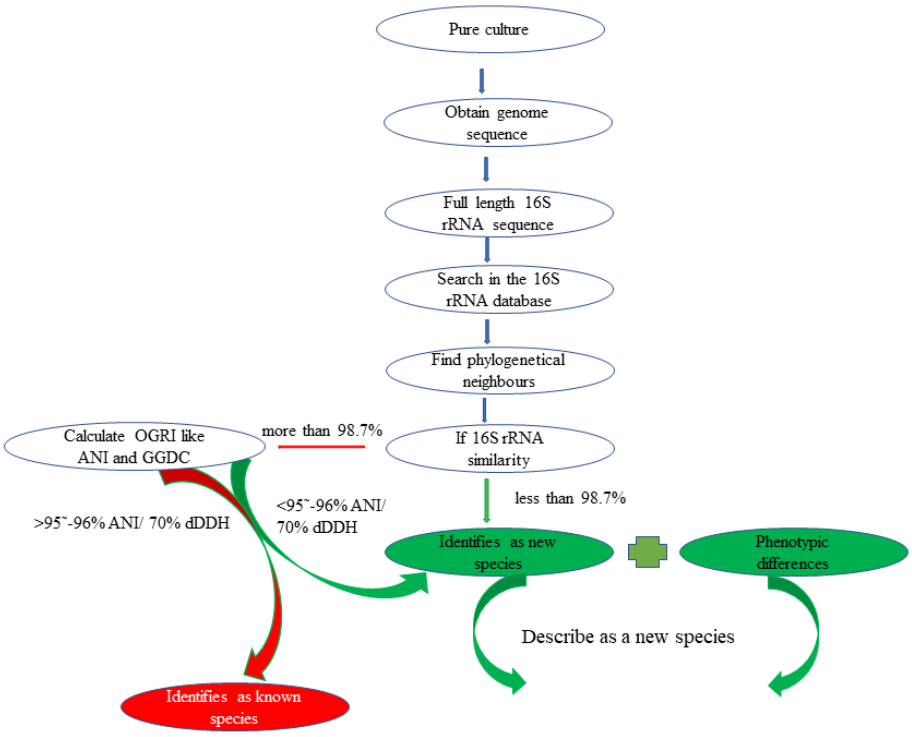


Fig. 1.1 Workflow of genome based polyphasic taxonomy at the species level. Data adapted from Chun et al. (2018).

1.3.1.3.1 Digital/*In-silico* DNA-DNA hybridization

Due to the errors, inability to build comparative database and laborious nature of conventional DDH, scientists were looking for the alternate methods. Advancement in the genome sequencing technology enabled the establishment of huge database of genome sequences which call the bioinformatics methods to calculate the *in-silico* DDH by comparing the genome sequences. The calculation *d*DDH involves three steps:

i. Determining the set of high-scoring segment pairs (HSPs) or maximally unique matches (MUMs) between the query and reference genomes

ii. Determining the distances between above sets

iii. Converting the distances into similarity percentage similar to DDH values.

As the *d*DDH values were well correlated with the 16S rRNA gene sequence identity and DDH values, 70% cut-off value maintained for species delineation between the two species (Auch et al., 2010a,b).

1.3.1.4 Average Nucleotide Identity (ANI)

ANI was first introduced by Konstantinidis and Tiedje (2005) by analyzing whole genome sequences of 175 strains and suggested that it would serve as an alternative method for error prone conventional DDH. ANI is the mean of identity values between the query and reference genomes' homologous region. The first step in calculation of ANI includes the fragmentation of query genome into 1020bp fragments. In the second step, each fragment is searched against the reference genome using the different algorithms like BLASTn and MUMmer. The mean identity of BLASTn matches are considered as ANI values between two organisms where the fragment should align at least 70% in length and should have more than 30% identity. The correlation studies between DDH and ANI revealed that the 95% ANI score corresponds to 70% DDH (Goris et al., 2007; Yoon et al., 2017). The ANI score calculation using by MUMmer was introduced (Richter and Rosselló Móra, 2009), which runs faster than ANIn (ANI calculated using BLASTn) and widely used (Kim et al., 2014; Rosselló Móra and Amann, 2015). But several studies suggested that ANIm (ANI calculated using MuMmer) is not suitable for calculation of ANI (Li et al., 2015). While calculating the DDH in classical method, it was known that reciprocal DDH values often vary (Tindall et

al., 2010). Similarly the ANI value of X to Y may differ from the Y to X. To overcome these, Lee et al. (2016) proposed the OrthoANI where the mean of ANI and reciprocal ANI was considered. Among the ANI calculating tools, ANIm and OrthoANIu run faster but for accuracy ANIb and OrthoANIu were the best (Yoon et al., 2017). The ANI score gave better results among closely related species.

1.3.1.5 Average Amino acid Identity (AAI)

If the ANI score between the species is less than 80% and/or 30% of their gene content is divergent, in that case AAI score can be calculated instead of ANI. AAI score are widely used in genus delineation instead of species. Based on suggestions of Goris et al. (2007) and Konstantinidis et al. (2017), two organisms with AAI score between 45-65% may be considered as belonging to different genera and those with score <45% considered as members of different families. However recent studies suggest that AAI values between members of related but different genera can vary between 60-80% (Luo et al., 2014; Orata et al., 2018). Apart from genus delineation, AAI score can be used to delineate species (Thompson et al., 2013).

1.3.1.6 DNA G+C mol%

In the prokaryotic taxonomy, variation in G+C mol% between two different species considered as an important taxonomic tool to differentiate them from each other. Proportions of guanine plus cytosine in the total nucleotides of genome is defined as G+C mol% of a given organism. It was considered in the bacterial genomes that G+C mol% vary between 20-80% (Rosselló-Mora and Amann, 2001), but recent studies suggest that the cut off is less than 20% (Nakabachi et al., 2006). DNA G+C mol% have been calculated using the indirect methods such as thermal denaturation, buoyant density in CsCl, melting profiles, HPLC (High

performance liqiuid chromatography), and real-time PCR (Meier-Kolthoff et al., 2014). Rapid improvement in the sequencing technologies allowed the estimation of G+C content directly from genome sequences by calculating the guanines and cytosines. Generally, the variation in G+C mol% within the species was considered to be 3-5% but Meier-Kolthoff et al. (2014) through genome sequencing re-estimated that it was less than 1%.

1.3.1.7 Melting temperature difference (Δ*T*m)

The temperature at which half of the double stranded DNA denatures is called melting temperature. This method is used as alternative method for DDH, where the DNA-DNA relatedness can be calculated as difference between the melting temperatures of reference strain and hybrid DNA. Even though, there is no direct transformation between percentage of binding and ΔT_m, but their results are well correlated (Gonzalez and Saiz-Jimenez, 2005). Several studies suggested that 70% DDH value corresponds to 5% Δ*T*m, hence, ≥ 5% Δ*T*m was recommended for species delineation (Gonzalez and Saiz-Jimenez, 2005).

1.3.1.8 Phylogenomics

Single gene based phylogenetic analysis often results in topological variation/conflicts (Castresana. 2007, Delsuc et al., 2005). MLSA was used to overcome this problem between closely related members (Whatmore et al., 2016 and Liu et al., 2017), but even in the MLSA the number of genes used, selection of genes and possibility of high variation between the genera was criticized by taxonomists and suggested that phylogenomics is the best to study the evolutionary relationships of taxa (Chan et al., 2012, Grana-Miraglia et al., 2018, Mateo-Estrada et al., 2019). Phylogenomics is defined as genome based phylogenetic tree inferred from the set of core genes (Eisen and Fraser, 2003). Based on the genomic data

availability, 37-104 core genes were identified by different research groups to construct phylogenomic trees (Creevey et al., 2011; Wu et al., 2013; Dupont et al., 2012; Na et al., 2018). PhyloSift (Darling et al., 2014), bcgTree (Ankenbrand and Keller, 2016), UBCG (Na et al., 2018) are the some examples for the phylogenomic tree softwares.

1.3.1.9 Pan-genome

Pan-genome is known as the repertoire of genes present in the analyzed dataset which consists of core genome (present in all the studied genomes), accessory genome (not core, not unique but present in few organisms) and unique genes (specific for strains) (Chen et al., 2018; Rouli et al., 2015). Pan-genome analysis has significance in evolution, pathogenicity, vaccine development and adaptations of bacteria (Chen et al., 2018). Pan-genome can be an open (if the new genome of strain added into the group then the size of pan-genome will increase) or closed (the size remains constant after some extant). Generally allopatric species have closed pan-genomes whereas sympatric species have open pan-genomes (Georgiades and Raoult, 2011; Diene, 2013). Taxonomy of bacteria based on core and pan-genome analysis is a powerful extension of the polyphasic approach (Inglin et al., 2018). Core genome consists of genes coding for essential processes like key metabolic pathways and processing of genetic information. Adaptations to specific environment, lateral gene transfer which leads to speciation were not found in the core genome (Inglin et al., 2018). Pan-genome analysis is helpful in studying the species/subspecies evolution. PGAweb (Chen et al., 2018), BPGA (Chaudhari et al., 2016), PGAP (Zhao et al., 2012) and Roary (Page et al., 2015) are some of the pangenome analysis softwares.

1.3.2 Phenotypic data

An organism's character is not just the genomic information present in it, rather it is also the expression genes and environmental interactions that will determine its phenotype. Before the emergence of genetic tools, classification of prokaryotes was solely based on phenotypic characters. The phenotypic characteristics helped in describing a taxon till genus and family level. The phenotypic traits include morphological, physiological and biochemical features of a particular organism. Phenotypic characterization gives predictability to the classification system, as the name of an organism can give insights to its characteristic features based on the taxa it belongs to.

Phenotypic classification depends upon the availability of pure cultures and laboratory facilities to cultivate the organisms. Since the expression of genes as phenotypes are highly influenced by the environmental conditions, a comparative analysis of phenotypic traits of putative novel taxa with their type strain is always preferred together (Tindall et al., 2010).

The major disadvantages with phenotypic classification are that it is laborious; time consuming as well as it is difficult to reproduce results which can only be ensured through highly standardized protocols (Rosselló Móra, 2009).

The phenotypic traits are those which are observed or analyzed under laboratory conditions like morphology, physiology and biochemistry of an organism. The morphological traits include colony morphology like colony shape, color, dimensions etc., the cellular characteristics like shape, Gram-staining properties, presence of endospore, flagella, inclusion bodies etc., The physiology and biochemistry of an organism is explained by observing growth at various

temperature, pH, salinity, oxygen conditions (aerobic or anaerobic), substrate utilization tests, enzyme activities, growth modes etc.

1.3.2.1 Chemotaxonomy

In chemotaxonomy, information of various cell constituents are collected and analyzed for classification of bacteria. The information obtained through chemotaxonomy will be the direct reflection of genetic expression of that organism in a particular point of time and environmental conditions. Chemotaxonomy comprises of all the information except the DNA and RNA information.

1.3.2.1.1 Polar lipids

Polar lipids constitute the major component among the numerous lipids present in bacterial membrane which aids great help in classification and identification purposes. Glycerophospholipids (phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol) are an important group of polar lipids, comprised two fatty acids linked to a glycerol molecule through ester bond, a phosphate group and a variable head group. Sulfolipids, glycolipids and aminolipids are some of the non-phosphorus membrane polar lipids. The use of bacterial polar lipids in taxonomy was initiated by Asselineau, O'Leary and kates (Lechevalier and Moss, 1977). Polar lipid analysis has been a central tool in chemotaxonomy and the presence/absence of glycolipid was considered as one of the genus differentiating characters in the reclassification of some species of the genus *Rhodobium* into a new genus, *Afifella* (Urdiain et al., 2008). The sphingophospholipids present in a constrained number of taxa are also important for classification (Jones and Krieg 1984). There are several methods for the extraction of polar lipids among which Bligh and Dyer (1959) and Kates (1986) methods are commonly used. Even though there is no recommendations to use the

two-dimensional thin layer chromatography (2D TLC) to separation and visualization of polar lipids, most of the taxonomists prefer to use 2D TLC (da Costa et al., 2011).

1.3.2.1.2 Fatty acid analysis

Fatty acids are mainly present in the membrane lipids and lipopolysaccharides, due to the uneven chain lengths, variable head group, double bond position, fatty acids composition has been using in bacterial taxonomy. Since, the fatty acid chain length, double bond position will change based on the environmental condition, analysis should be done under identical conditions unless the query and reference strains cannot grow under same condition. Myron Sasser developed a method that labeled whole cell fatty acids of bacterial cells that could then be analyzed by an automated gas chromatography (GC), now this technique calling as Sherlock® microbial identification system (MIDI Inc.) (Kunitzky et al., 2005). The MIDI Inc. is used to identify fatty acid composition based on the retention time, in which fatty acids were hydrolyses from various membrane lipids and then add the -CH_3 group to the -COOH group, which form methyl esters (fatty acid methyl esters analysis in short FAMEs) which act as a label for gas chromatography. In general phylogenetically related members have similar kind of fatty acids. Initially MIDI Sherlock® microbial identification system was developed to identify the bacteria rapidly, but now it is employed to study bacteria, fungi and some nematodes fatty acid analysis (Sekora et al., 2009). For specific group of bacteria specific libraries were made is available in MIDI Inc., for example RTSBA6, TSBA6 for aerobic bacteria, BHIBLA for anaerobic bacteria, ACTIN1 for Actinobacteria.

1.3.2.1.3 Respiratory Quinones

All living organisms possess respiratory quinones except for few obligatory fermentative bacteria and methanogens. However, *Methanosarcina mazei* (member of order *Methanosarcinales*) synthesize methanophenazine which act as substituent for electron carrier, (Elling et al., 2016). Quinones are key players in electron transport, oxidative phosphorylation and active transport. (Collins and Jones, 1981). Mainly two types of quinones i.e naphthoquinones (phylloquinones and menaquinones) and benzoquinones (plastoquinones and ubiquinones) are found. Apart from these two, rhodoquinone and caldariellaquinone are also reported (Glover and Threlfall, 1962; de Rosa et al., 1977). Most of the bacteria possess menaquinones or ubiquinones but, they will differ in the number of isoprene units (Li, 2010). Generally, strict anaerobic bacteria possess menaquinones than ubiquinones, whereas Gram-negative strict aerobes possess ubiquinones than menaquinones (Li, 2010). Glover and Threlfall (1962) reported the rhodoquinone production by *Rhodospirillum rubrum* (Collins and Jones, 1981) and later it was also discovered in some animal species (Van Hellemond et al., 1996). Till now only one gene i.e., *rquA* involvement in rhodoquinone synthesis is known (Lonjers et al., 2012). Initially the role of *rquA* gene was unclear but Bernert et al. (2019), revealed that it can convert ubiquinone to rhodoquinone in *Rhodospirillum rubrum*. But recently, Borrello et al. (2019) reported *Caenorhabditis elegans* can produce rhodoquinone using precursor of kynurenine pathway. Since, none of the hosts produce the rhodoquinones, it could be a potential target for inhibiting the parasites like *Fasciola hepatica* (Borrello et al., 2019). Due to the structural diversity of quinones, diversity in the isoprene units it possess, saturated/unsaturated state of isoprene units, quinone identification is important in

polyphasic taxonomy. Studies revealed that the bacterial diversity can be studied by analyzing the quinone profiling from environmental sample (Kunihiro et al., 2014).

1.3.2.1.4 Pigment analysis

Sometimes, the pigmentation of colony/culture immediately connected to the way of life of the cells. Especially in phototrophic bacteria, pigments play a key role, to convert light energy into chemical energy. Mostly, pigmentation of culture and degree of pigmentation vary based on the environment and growth media, sometimes the pigmentation is species specific (Oren, 2011). Bacteriochlorophyll (BChl) *a* and carotenoids characterization is important in photosynthetic bacterial taxonomy, and their presence can be detected by whole cell absorption spectra.

1.3.2.1.4.1 Whole cell absorption spectra

The presence of pigments within or outside cell (excreted out by the cell) can be detected by whole cell absorption spectrum either by using intact cells or by using extracts of pigments in suitable solvent. These pigments can be detected in the wavelength from 300 nm to 1100 nm. Due to the environment condition, the *in vivo* absorption of pigments and the solvent extract pigments from same cells will differ and changes are given in the Table 1.1 (Oren, 2011).

Table 1.1. The Bacteriochlorophylls absorption maxima in organic solvent and intact cells (Data taken from Oren, 2011; Overmann and Garcia-Pichel, 2006)

Type of Chlorophyll	***In-vivo* absorption maxima (nm)**	**absorption maxima in organic solvent (ether) (nm)**
BChl *a*	830-910	770
BChl *b*	986-1,035	790
BChl *c*	745-755	660
Chlorophyll *a*	680-683	665
BChl *d*	715-745	650
BChl *e*	710-725	
BChl *g*	788-790	763

1.3.2.1.4.2 Bacteriochlorophylls

Oxygenic phototrophic bacteria (Phylum *Cyanobacteria*) possesses chlorophyll *a* and have absorption maxima of intact cells at 680-683 nm. Anoxygenic phototrophic bacteria possess different types of bacteriochlorophylls, BChl *a* is found in most of them. Till now, some *Acidiphilium* members are reported to have zinc containing BChl *a*, whereas all other photosynthetic bacteria have magnesium containing BChl *a* (Overmann and Garcia-Pichel, 2006). BChl/Chl, carotenoids and some protein all together form photosynthetic membranes i.e., thylakoids of chloroplasts and intracytoplasmic membranes (vesicles/lamellar/tubular) in bacteria. In bacterial taxonomy, presence/absence of BChl, type of BChl, type of intracytoplasmic membrane has greater importance in species delineation.

The diversity and phylogenetic relationship of the photosynthetic bacteria (mostly α-Proteobacteria and β-Proteobacteria) can be studied using the genes present in photosynthetic gene cluster (PGC), especially reaction center proteins encoded genes like *pufL* and *pufM* (Tank et al., 2009). The organization of genes in PGC is also considered as important taxonomic marker. By comparing the closely related members it was reported that PGCs loss from the bacterial genomes, which further shifted from phototrophic growth mode to chemotrophic growth mode (Zheng et al., 2012). In contrast to above statement several studies suggest that, the PGCs can be transferred from one bacterium to other bacterium via horizontal gene transfer (Brinkmann et al., 2018). Recent study revealed the patchy distribution of PGCs among *Roseobacter* clade, is due to the loss or gain of PGCs through extra chromosomal replicons (ECRs) (Liu et al., 2019).

1.3.2.1.4.3 Carotenoids

Carotenoids are well distributed in the bacteria and are responsible for the color of some bacteria. Carotenoids absorb the high energy zone (400 to 500 nm) of visible range and transfer the energy to BChl/Chl in the reaction center. They protect the cells from photodynamic damage (Oren, 2011), They also involve in the prevention of several human diseases like acute myocardial infarction (Koh et al., 2011), many types of cancers (Tanaka et al., 2012; Wertz et al., 2004; Grubbs et al., 1991) and helps to decrease the low-density lipoprotein (LDL) cholesterol level (Fuhrman et al., 1997). More than 750 different naturally existing carotenoids are identified (Zaghdoudi et al., 2017). Structurally carotenoids are divided into two main groups i.e. Carotenes (present carbon and hydrogen) and Xanthophylls (present carbon, hydrogen and oxygen in the structure). The carotenoids diversity is more among the Anoxygenic photrophic bacteria (APBs) and has mainly five different types of pathways for carotenoid production i.e. spirilloxanthin pathway, okenone pathway, isorenieratene pathway (isorenieratene, chlorobactene), γ- and β-carotene pathway and diapocarotene pathway (Takaichi, 1999).

Carotenoid content may vary based on the culture age, light intensity and environmental conditions. Due to these reason, carotenoid identification of newly isolated and reference strain should be test under identical conditions. Imhoff and Caumette (2004) stated that carotenoid identification cannot be done using the *in vivo* absorption spectra and identification was optional property for phototrophic bacterial species description. However many taxonomists employ high performance chromatography (HPLC) to identify the carotenoid present in the novel isolates (Lakshmi et al., 2011; Divyasree et al., 2015; Hiraishi, 2017).

1.4 Anoxygenic phototrophic bacteria

The "Anoxygenic phototrophic bacteria" (APB) encompasses an extremely heterogeneous physiological group of prokaryotes that are capable of converting radiant energy into chemical energy under anaerobic conditions but do not release oxygen unlike cyanobacteria. The heterogeneity of APB is demonstrated in their habitats, morphology, physiology, radiation capturing pigments and phylogeny. Anoxygenic photosynthesis is catalyzed by a single photosystem, wherein electrons travel through a closed circuit of an electron transport chain. Here an external supply of electrons comes from sulfide, other reduced sulfur compounds, hydrogen or organic molecules (Heising and Schink, 1998). The first anoxygenic phototrophic bacteria was described by Molisch in early 20th century, which belongs to *Rhodospirillum* species, and at almost at same time Nadson (1906) described another anoxygenic phototrophic bacteria *Chlorobium limicola.*

Anoxygenic photosynthesis is widely distributed over several bacterial phyla, including Chlorobi (commonly called as green sulfur bacteria), Chloroflexi (green non sulphur bacteria), Proteobacteria, Firmicutes (Gram-positive Heliobacteria), Acidobacteria (*Chloracidobacterium thermophilum*) and Gemmatimonadetes (*Gemmatimonas phototrophica*) (Hanada, 2016). Though the common property of anoxygenic photosynthesis places the members of APB in one physiological group, 16S rRNA gene based classification identified them as separate evolutionary lineages (Imhoff et al., 2019).

The oxygen producing organisms (cyanobacteria, algae and plants) share common properties like presence of chlorophyll *a* and photosystem I and II. But, APBs shows more diversity among themselves in terms of photosynthetic apparatus, bacteriochlorophyll (Bchl) content and photosystem i.e., either one or

two. Distribution of photosystem I or II in the APBs and their absorption maxima is given in Table 1.2. Having the different absorption light band lengths for different APBs, they all can co-exist in a same niche (Hanada, 2016).

Table 1.2. Distribution of APBs in different phyla and type of photosystem they comprise (Hanada, 2016; Tank and Bryant, 2015; Zeng et al., 2015)

Phyla	**Type of photosystem**	**Absorption maxima (nm)**
Chlorobi Firmicutes Acidobacteria	Photosystem I (Fe-S type reaction centers)	740–750 (chlorosome) 786–792 740–750 (chlorosome)
Chloroflexi Proteobacteria Gemmatimonadetes	photosystem II	740–750 (chlorosome) 800–1020 800–1020

1.4.1 Phototrophic Proteobacteria

Majority of phototrophic Proteobacteria grow anaerobically under photoheterotrophic/photoautotrophic conditions, while chemotrophy is less common and occurs under aerobic conditions. These bacteria have BChl *a*/ BChl *b* and type II reaction center (Zeng et al., 2014). Almost a century back, Molisch (1907) differentiated the phototrophic Proteobacteria into purple sulphur bacteria (which can deposit sulphur granules through oxidation of sulphur and maximum members are photoautotrophic in nature) and purple non sulphur bacteria (have maximum members with photoheterotrophic life style using different organic compounds as electron donor and carbon source and can't tolerate the suphide concentration as purple sulphur bacteria). In the phylum Proteobacteria, phototrophic purple sulphur bacteria (PSB) are distributed within the class Gammaproteobacteria, while the phototrophic purple non sulphur bacteria (PNSB) with the class Alphaproteobacteria and Betaproteobacteria (Imohoff, 2017).

However, the names PSB and PNSB were based on the physiological feature of bacteria, now these names are redundant.

Aeorobic anoxygenic phototrophic bacteria are another group of Proteobacteria, possess photosynthetic apparatuses but can't support growth by photosynthesis. *Erythrobacter longus* (Shiba and Shimidu, 1982) was first identified bacterium in this group followed by *Roseobacter litoralis* and *Roseobacter denitrificans* (Shiba, 1991).

In the phylum Proteobacteria more than 160 phototrophic anoxygenic phototrophic bacterial taxa are distributed in Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Imhoff, 2017).

1.4.1.1 Family 'Rhodobacteraceae'

The family 'Rhodobacteraceae' belongs to the class Alphaproteobacteria was described by Garrity et al. in 2005 and it can be considered as the model taxa for modern taxonomy (Pujalte et al., 2014). Presently contains more than 150 validly named genera (http://www.bacterio.net/). However, it must be noted that the name 'Rhodobacteraceae' is illegitimate (http://www.bacterio.net/rhodobacteraceae.html) as the relationship between this suprageneric grouping and the family *Hyphomonadaceae* (Lee et al., 2005) has not yet been resolved. Most of the 'Rhodobacteraceae' members are aquatic, comprising both phototrophs, chemotrophs and exhibit phenotypic, metabolic and ecological diversity. Mostly they are marine in nature and require NaCl or combination of salts.

They are Gram-stain-negative cells, multiplication occurs via budding or binary fission, some show motility usually with polar flagella. Most of them (chemotrophs and phototrophs) have carotenoids of spheroidene series in addition

to Bchl *a*. Commonly store carbon as polyhydroxybutyrate and few other store polyhydroxyalkanoates. Q10 is the major or the only respiratory quinone, $C_{18:1}\omega7c/C_{18:1}\omega6C$ are the major fatty acid, phosphatidylcholine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine are the major polar lipids. The mol% G+C content varies between 47-76%.

Lee et al. (2005) grouped members of the family 'Rhodobacteraceae' into five-well defined groups on the basis of their 16S rRNA gene phylogenetic analysis. These are the *Rhodobacter*, *Rhodovulum*, *Amaricoccus*, *Roseobacter* and *Paracoccus* groups. These groups are metabolically, physiologically and ecologically different and give base for their suggested recognition as new families (Pujalte et al., 2014). The *Roseobacter* group is large group that harbous half of the genera present in *Rhodobacteraceae.* Most of the members were isolated from marine habitats and some members exhibit aerobic anoxygenic phototrophy. Most of the members have the ability to degrade dimethylsulfoniopropionate (DMSP) and have ability to do the oxidation of carbon monoxide (González et al., 2000; Wagner-Döbler and Biebl, 2006). These organisms can utilize methylated amines as alternative nitrogen source (Chen 2012; Pujalte et al., 2014).

The *Paracoccus* group harbours two genera; *Paracoccus* which have more than 50 valid species names and the *Methylarcula* which have only two valid species names (Pujalte et al., 2014). Members of *Paracoccus* group shows chemotrophic mode of growth with some members have denitrification capability. Phylogenetic and phylogenomics analysis revealed the close relationship between *Rhodobacter* group and *Paracoccus* group (Gupta and Mok, 2007). The *Amaricoccus* group is distinctly related group to the rest of the family and possesses APBs, AAPBs and chemotrophs. The *Rhodovulum* group members are

marine and halophilic and require salt requirement for their optimum growth and are dominated with the PNSB members. The *Rhodobacter* group members were isolated from freshwater habitats with few exceptions. The *Rhodobacter* group consists of 17 genera. Out of these seventeen genera, members of *Cereibacter*, *Rhodobaca* and *Rhodobacter* genera perform anoxygenic photosynthesis.

1.4.1.1.1 Genus *Rhodobacter*

The genus *Rhodobacter* (*Rba*.) was proposed by Imhoff et al. (1984) to accommodate the *Rhodopseudomonas* species (*Rhodopseudomonas capsulata*, *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas sulfidophila* and *Rhodopseudomonas adritica*) which have vesicular intracytoplasmic membranes as *Rba. capsulatus* (type species), *Rba. sphaeroides*, *Rba. adriaticus* and *Rba. sulfidophilus.* However, based on 16S rRNA gene sequence comparisons, distance matrix analysis, genomic DNA-DNA hybridization data, salt requirement for optimal growth, sulfide tolerance, final oxidation product of sulfide, and polar lipid composition. *Rba. sulfidophilus*, *Rba. euryhalinus*, *Rba. adriaticus* and *Rhodopseudomonas blastica* were subsequently reclassified as *Rhodovulum sulfidophilum*, *Rhodovulum euryhulinum*, *Rhodovulum adriaticum* and *Rhodobacter blasticus*, respectively (Hiraishi and Ueda, 1994). The genus description of *Rhodobacter* and present status is given in the Fig. 1.2.

Members of the genus *Rhodobacter* perform anoxygenic photosynthesis, fix dinitrogen and play a key role in bio-geochemical cycles and are model organisms for protein expression studies (Jaschke et al., 2011), green energy studies (Ravi et al., 2019), bio-active compounds production (Troost et al., 2019), studies on adaptation strategies of bacteria to metal stress (Volpicella et al., 2014), as well as bioremediation (Bin et al., 2004).

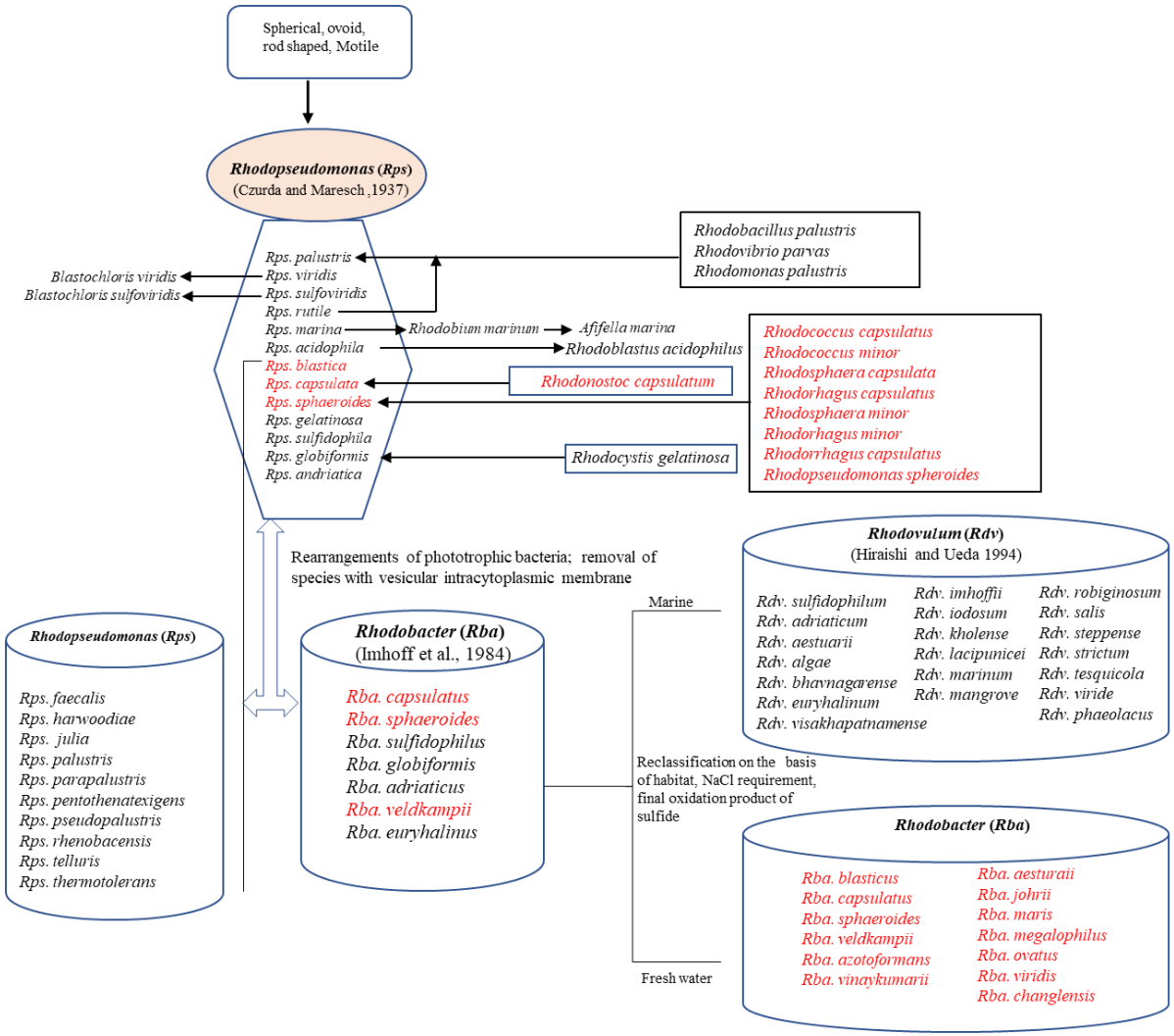


Fig. 1.2 Graphical representation of phototrophic bacteria rearrangements, emphasizing on genus *Rhodobacter*. Data taken from van Niel (1944); Imhoff et al. (1984); Hiraishi and Ueda (1994) and from LPSN (https://www.bacterio.net/)

During the initiation of this study the genus *Rhodobacter* (*Rba.*) comprised of 12 valid species names: *Rba. aestuarii*, *Rba. azotoformans*, *Rba. blasticus*, *Rba. capsulatus*, *Rba. johrii*, *Rba. maris*, *Rba. megalophilus*, *Rba. ovatus*, *Rba. sphaeroides*, *Rba. veldkampii*, *Rba. vinaykumarii* and *Rba. viridis*. Members are ovoid to rod-shaped, Gram-stain negative, facultative photoheterotrophs and have vesicular/lamellar intra cellular membrane (ICM) architecture, bacteriochlorophyll-*a*, carotenoids of the spheroidene series and Q10 as their major quinone. Some of the members are capable of growing photolithoautotrophically in the presence of sulfide/H_2 as an electron donor (Imhoff, 2005; Girija et al., 2010, Suresh et al., 2017; Gandham et al., 2018). *Rhodobacter* members are widely distributed in oxic/anoxic zones of aquatic habitats (Hansen and Imhoff 1985; Suresh et al., 2017), mud (Shalem Raj et al., 2013; van Niel, 1944), sediment (Subhash and Lee 2016; Srinivas et al., 2008, Suresh et al., 2019a), agricultural field (Girija et al., 2010), estuarine habitats (Venkata Ramana et al., 2008, 2009), sludge (Hiraishi et al., 1996), marine habitats (Srinivas et al., 2007) and hot spings (Xian et al., 2019; Khan et al., 2019).

Based on 16S rRNA gene sequence phylogenetic analysis, we could observe heterogeneity among the members of the genus *Rhodobacter*, due to a large diversity of interspersing chemotrophs dividing the members into five monophyletic clusters (Suresh et al., 2017). Apart from being polyphyletic, the members of the genus *Rhodobacter* also have phenotypic differences which mainly include heterogeneity in polar lipids (presence and absence glycolipids/diphosphatidylglycerol [DPG]), ICM architecture (vesicular/lamellar), sulfur metabolism and cell division (budding/binary fission). The phenotypic diversity among the taxa of the genus *Rhodobacter* was critically commented upon

2½ decades ago (Hiraishi and Ueda, 1994). However, due to a lack of strong evidence, reclassification of this genus has not yet been taken up. Taxogenomics (phylogenomics) helped in similar reclassification of members of *Mycobacterium* (Gupta et al., 2018), *Phaeobacter* (Breider et al., 2014), Gammaproteobacterial methanotrophs (Orata et al., 2018), *Geobacillus* (Aliyu et al., 2016), *Burkholderia* (Lopes-Santos et al., 2017), *Populibacter* (Li et al., 2017) and *Roseobacter* group (Wirth and Whitman, 2018).

The present study was carried out to isolate, describe (novel taxa if any) of the genus *Rhodobacter* and to resolve the taxonomic conflict within the genus.

1.5 Objectives

1. To isolate *Rhodobacter* spp. and describe novel taxa, if any, using the polyphasic taxonomic approach
2. To resolve the taxonomic conflict within the genus *Rhodobacter*

Material and methods

2. Materials and Methods

2.1 Glasswares

Glasswares which were used includes petriplates, Erlenmeyer flasks, measuring cylinders, round bottom flasks, screw cap test tubes and bottles of different brands like Borosil, Anumbra, Rivera and Scott Duran.

2.2 Cleaning

All required glasswares were cleaned with 'Teepol', soaked in chromic acid solution for a day, cleaned with tap water, further rinsing was done with distilled water and dried in the oven before used.

2.3 Distilled water, double distilled water and Milli-Q water

Media preparation was done routinely by distilled water, buffer preparations by double distilled water, DNA, RNA works by Milli-Q water. All the three kind of water were obtained from Millipore facility at School of Life sciences, UoH.

2.4 Chemicals and solvents

Analytical grade chemicals, solvents and kits of Merck, Sigma-Aldrich, Genetix, HiMedia were used in this study.

2.5 Gases used

Hydrogen, Nitrogen, Argon of 99.9% purity were used for the present study and commercial grade supplied by Goyal gasses Pvt. Ltd., Hyderabad

2.6 Determination of pH

Digisun Electronics (DI-707) was used for checking the pH.

2.7 Sterilization

Sterilization were done either by autoclaving (heat tolerant materials and media) or by filter sterilization (heat labile compounds) using 0.2µm acrodisc syringe filter manufactured by Pall Life Sciences.

2.8 Sample collection

Samples including soil, water and algae were collected from Gujarat and other parts of India in polythene zip-lock covers and sterile centrifuge tubes (15ml or 50ml). The GPS locations of the site, pH and salinity (using AD310 conductivity meter made by Adwa instruments, Romania which measures electrical conductivity; then converted into salinity percentage) were noted during sample collection. Within 10 days of sample collection, samples were kept for enrichment using suitable media and the rest of samples were refrigerated for further analysis.

2.9 Enrichment and isolation of *Rhodobacter* members

2.9.1 Enrichment using the transparent glass bottles

Approximately 0.2 ml of water or 0.2 g of the sediment sample was inoculated in 45 ml transparent screw cap glass bottles which were filled with the modified Biebl and Pfennig's media (MBP) (Lakshmi et al., 2011; Table 2.1) with pyruvate as a carbon source and electron donor for enrichment. The NaCl concentration was increased to 2% in the same media for the enrichment of marine samples.

2.9.2 Paraffin wax method

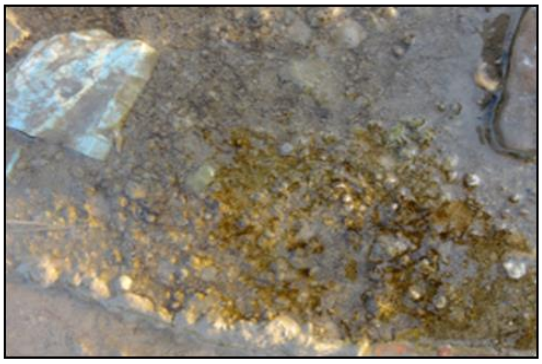
The modified Biebl and Pfennig's (MBP) media was prepared by using 1.8% - 2.0% agar as solidifying agent. Approximately, 25ml of this media was transferred into a sterile 60ml test tube and when it reached 40 °C temperature, As inoculum, 0.2 ml of water sample (for sediment samples, 0.2 g of sediment was dissolved in 10ml of sterile water) was added to the test tube containing media. This media was transferred to petriplates after proper shaking and allowed to solidify. Then paraffin wax (when it cooled down to 50 °C) was added to that plate, which would solidify in few minutes. therefore making a solid layer above the agar layer ensuring anaerobic condition (Archana et al., 2004).

2.10 Some of the photographs of sampling site (Fig. 2.1)

Sample Name: GJ1: Location: **Bhavnagar**
Sample type: Sediment
GPS location: N 21° 700' E 72° 228'

Sample Name: GJ6: Location: **Bhavnagar**
Sample type: Sediment
GPS location: N 21° 700' E 72° 228'

Sample Name: GJ21: Location: **Nari salt pan;** Sample type: Sediment
GPS location: N 21° 797' E 72° 069'



Sample Name: GJ30B: Location: **Alang Ship yard;** Sample type: Sediment
GPS location: N 21° 442' E 72° 225'

Sample Name: GJ42, Location: **Mahemdavad**
Sample type: Fresh water pond filled with azolla
GPS location: N 22° 474' E 72° 431'

Sample Name: GJ64: Location: **Jafrabad**
Sample type: Sediment
GPS location: N 20° 55' 39" E 71° 24' 43"

Continued

Sample Name: GJ77; Location: **Diu**
Sample type: Sediment
GPS location: N 21° 716' E 72° 960'

Sample Name: GJ95; **Porbander**
Sample type: Sediment
GPS location: N 21° 65' E 69° 60'

Sample Name: GJS3; **Next to Porbander**
Sample type: Sediment
GPS location: N 21° 63' E 69°58'

Sample Name: GJS6; **Nandanvan**
Sample type: Sediment
GPS location: N 22°26' E 69° 06'

Sample Name: GJ52; **Gopnath**
Sample type: Brown layer on water
biofilmS location: N 21° 210' E 72° 109

Sample Name: GJS20; **Kandla**
Sample type: Sediment+ Purple water
GPS location: N 23° 01' E 70° 12'

Table 2.1 Modified Biebl and Pfennig's (MBP) media composition

S. No	Media Component	grams/Litre
1	$CaCl_2.2H_20$	0.05
2	$MgSO_4.7H_2O$	0.2
3	Yeast extract	1.0
4	NaCl	0.4
5	KH_2PO_4	0.5
6	NH_4Cl	0.6
7	Sodium pyruvate	3.0
8	Ferric citrate	(5 ml from a 0.1 % w/v stock)
9	Peptone	1.0
10	Vitamin B_{12} *	1ml from a 0.2mg/10ml stock
11	SL7	1 ml

*Filter sterilized solution was added after autoclave

SL7 contain following (mg/L)

	SL7 components	mg/L
1	$CoCl_2.6H_2O$	200
2	$MnCl_2.4H_2O$	100
3	$NaMoO_4.2H_2O$	40
4	$ZnCl_2$	70
5	H_3BO_3	60
6	$CuCl_2.H_2O$	20
7	$NiCl_2.6H_2O$	20
8	HCl	25 % v/v; 1 ml

2.11 Purification

Rhodobacter members were purified from enrichment cultures by continuously streaking on agar plates/agar on the same MBP media. The slants were made in 25 x 150 mm test tubes by adding 20 ml of media and it was flushed with Argon gas to establish anaerobic condition. Streaking was repeated until identical colonies appeared on two successive slants of same culture. To check the purity, the cultures were streaked on nutrient agar (HiMedia) plates and incubated at 30-35 °C

temperature under the illumination of 2,400 lux. The colonies were observed frequently for their colour, morphology and appearance under microscope in order to prevent other phototrophic bacterial contamination.

2.12 Preservation of stock cultures

2.12.1 Glycerol stocks

Purified *Rhodobacter* members were maintained as glycerol stocks. 50% glycerol stocks were made by adding 1ml of culture (MBP media) in 1ml of 100% glycerol. The stocks were stored at 4 °C for overnight and shifted to -20 °C for long term use.

2.12.2 Agar stabs

In 5ml centrifuge tube, the MBP media with 1.8% agar was added up to three quarters of the test tube. The cultures were stabbed into the media, and kept for incubation at 30-35 °C temperature under the light. After allowing it to grow for 4-5 days, it was transferred in 4 °C for long term use. Sub-culturing of stabs were done periodically in 90 days and checked for contamination.

2.12.3 Lyophilisation

Rhodobacter members were maintained in the form of lyophilized pellets in sterile vials and stored at 4 °C. Anaerobically grown *Rhodobacter* members on agar slants with MBP media with 1.8% agar were incubated under 2400 lux illumination and 30±2 °C temperature, until late log phase. Seven to eight millilitre of cry protectant (10% sterilized skimmed milk) was added into the tube and scraped using the loop to make bacterial suspension of 108 to 109 CFU/ml concentration. 300 µl of this cell suspension was transferred to glass ampoules and incubated at -70 °C for overnight and then subjected to primary drying (≤40 °C and ≤100 mT vacuum for 6 to 8 h). After that the secondary drying was done (≤40 °C and ≤100 mT for 2 to 4 h).

The dried glass ampoules with culture were sealed by oxygen torch under vacuum conditions. Spark tester was used to check the leakage in sealed ampoules. Good condition ampoules were labelled and stored at 4 °C. The viability of the culture was checked by reviving the lyophilized culture. Reviving of culture was done as follows; one ampoule was tested by adding 300 µl of Biebl and Pfenning's media into it. After 30 min incubation it was transferred to a screw cap tube containing MBP media (media was filled till the rim of tube) and incubated under illumination of 2,400 lux at 30-35 °C temperature.

2.13 Morphological characterization

2.13.1 Morphological observation

Colony morphology was observed in the MBP agar medium at optimum growth condition.

2.13.1.1 Microscopic observation

Morphological characters like cell size, cell shape, cell motility and cell division were observed using phase contrast microscope (Olympus- B201).

2.13.1.2 Scanning electron microscopy (SEM)

Two millilitres of log phase culture was centrifuged at 6000g for 15 min at 25 °C. The resulted cell pellet was washed by suspending in phosphate buffer (0.05mM, pH 7.2) and centrifuged at 6000g for 10 min at 25 °C. The pellet was re-suspended in 0.25% 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- 100% (10% interval) and finally the cells were dissolved in 100% ethanol. Sample was kept on the small size coverslips which was kept on the SEM stab with help of adhesive tape. Finally, SEM stabs were kept for gold sputtering and then viewed under the SEM (Philips XL3O)

2.14 Staining methods

2.14.1 Gram staining:

Gram staining was done using the HiMedia Gram's stains kit, according to the manufacturer instructions.

2.14.2 Negative staining for Transmission Electron Microscopy (TEM)

2% of uranyl acetate was used as a negative stain. The staining procedure is as follows:

10µl of log phase culture was added to the 200 mesh carbon coated copper grid (TED PELLA, INC.). After 2 min the extra culture was removed by touching the edge of grid with the Whitman filter paper. In the same way 10µl negative stain was added to the grid and after 2 min extra stain was removed. After 10 min of air dry the grids were visualized under TEM microscopy (H-7500 Hitachi).

2.14.3 Transmission electron micrograph sections for ICM structures

Ultrathin sections of bacteria were outsourced at RUSKA lab, Hyderabad. Two millilitres of log phase culture was centrifuged at 6000g for 15 min at 25 °C. The resulted cell pellet was washed by suspending in phosphate buffer (0.05mM, pH 7.2) and centrifuged at 6000g for 10 min at 25 °C. The pellet was re-suspended in 0.25% glutaraldehyde solution and kept for overnight incubation at 4 °C. The glutaraldehyde solution was replaced with 2% aqueous osmium tetroxide and then the dehydration of samples were done by ethanol (increased concentration of ethanol from 10- 100%; 10% interval). Samples were infiltrated and embedded in Spurr's resin. With the help of ultramicrotome (Leica-UCT-GA-D/E-1/100) bacterial sections were made and stained with uranyl acetate. 4% lead citrate used as a counter stain. Finally the sections of bacteria mounted on cooper grids and observed under TEM (H-7500 Hitachi).

2.15 Biochemical characterisation

2.15.1 Catalase and oxidase test

48 hours grown culture (one loopful of cells) was transferred into fresh glass slide and one to two drops of 3% H_2O_2 was added on the pellet. Immediate bubble formation would show positive result. Catalase positive and negative cultures were taken for the confirmation. The oxidase test was done using Oxidase disc (HiMedia) according to the instructions of manufacturer.

2.15.2 Nitrate reduction test

Nitrate reduction test was carried out using the HiMedia disk according to the manufacturer instructions.

2.15.3 Organic carbon utilization tests

Various carbon compounds (glucose, rhamnose, fructose, acetate, ascorbate, cysteine, ethanol, fumarate, glycerol, lactate, malate, succinate, sucrose, tartrate, lactose, glutamate, D-sorbitol, D-mannose, D-mannitol D-galactose, Tween 80 and D-cellobiose) utilization were tested by replacing the pyruvate in MBP media.

2.15.4 Physiological characterization

Growth of bacteria was measured turbidimetrically (optical density) using the colorimeter (Systronics make, model 112) at 660 nm (filter 8) against blank (un-inoculated medium). Increase in the optical density considered as increase in the bacterial growth.

2.15.5 Utilization of various nitrogen sources

MBP media was to check the utilization of various nitrogen source (sodium nitrite, glutamine, urea, NH_4Cl, $NaNO_3$ and glutamate 0.07%, w/v). Growth was measured at 660 nm using the control (without any nitrogen source).

2.16 Growth modes

2.16.1 Phototrophic growth mode

Phototrophy of organisms were checked in transparent glass screw cap test tubes (10 X 100 mm) using the MBP medium at 30±2 °C. The photolithoautotrophic growth mode was tested in the light (2400 lux) in anaerobic condition, with $Na_2S_2O_3.5H_2O$ (5mM)/sulfite (2mM)/$Na_2S.9H_2O$ (2mM) as electron donor and $NaHCO_3$ as carbon source. Pyruvate was used as electron donor and carbon source check the photoorganotrophic growth mode.

2.16.2 Chemotrophic growth mode

Chemotrophic growth modes were checked in Erlenmeyer flask using the MBP media aerobically using the electron donor (5mM $Na_2S2O_3.5H_2O$) and carbon source (0.1 %, w/v $NaHCO_3$) and incubated in shaker (150 rpm at 30+2 °C). Pyruvate was used as electron donor and carbon source to check the chemoorganoheterotrophic growth mode.

2.16.3 Fermentative growth mode

0.3% (w/v) pyruvate/glucose/fructose as carbon source in MBP media was used to check the bacterial fermentative growth mode. The culture was inoculated in fully filled transparent glass test tubes and incubated in dark at 30-35 °C.

2.17 Vitamin requirement

Bacterial culture was inoculated in MBP media by replacing the yeast with the filter sterilized (0.22 µm Acrodisc syringe filter) vitamin solutions (Biotin [50 µgl-1], nicotinic acid [300 µgl-1], thiamine [300 µgl-1], calcium pantothenate [10 µgl-1], *para*-aminobenzoic acid [200 µgl-1], B_{12} [15 µgl-1], pyridoxal phosphate [15 µgl-1]). Repeated subculture without the vitamins was carried out for three subsequent transfers to determine the absolute requirement.

2.18 Sodium chloride requirement for optimum growth

The mineral medium was prepared with NH_4Cl (0.07 %, w/v) as nitrogen source, in which different concentrations of sodium chloride (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 %) were added separately in each tube prior to autoclaving. The inoculated cultures were incubated at 30-35 °C.

2.19 Growth at different temperatures

The culture in the MBP media was incubated at various temperatures (15, 20, 25, 30, 35, 40 and 50 °C) and culture growth was observed.

2.20 Growth at different pH

Instead of water in MBP media with optimum salinity, K_2HPO_4-KH_2PO_4 buffer for pH 5 -8, and $NaHCO_3$-NaOH buffers for pH 9-11 were used to check the growth of culture in different pH and after inoculation, incubated in optimum temperature to monitor culture growth.

2.21 Chemotaxonomic characterisation

2.21.1 Pigments analysis

2.21.1.1 Whole cell absorption spectrum

This was done according to the Trüper and Pfennig's (1981). To the 3.5 ml of 3-4 days old broth, 5 grams of sucrose was added and vortexed properly. Absorption spectrum of 300-1100 nm range was measured (Spectronic Genesys 2 spectrophotometer) using the MBP medium with sucrose as blank.

2.21.1.2 Identification of carotenoids by HPLC

The cells grown in photoheterotrophic, anaerobic condition were centrifuged at 7,000 rpm at 25 °C for 10 minutes. Wet pellet (0.5 g) pigments were extracted into acetone and methanol (7:2) by vortexing for 5 minutes. Then it was concentrated using rotary evaporator and analysed through HPLC. Acetonitrile: methanol:

tetrahydrofuran (58:35:7) solvent system, at a flow rate of 1.5 ml/min at room temperature using Shimadzu SPD 10AVP isocratic system, Luna 5µm C18 100A column (250 x 4.6mm) and PDA detector at 450 nm were used to detect the compounds and a range of wavelength was also set between 190-800 nm. Total run time was 20 minutes.

2.21.2 Cellular fatty acid composition

Gas chromatography was used to analyse the cellular fatty acid methyl esters based on the instructions of MIDI Inc. (Sasser, 1990), which was done by outsourcing to Royal Life Sciences Pvt. Ltd., Secunderabad.

2.21.3 Polar lipid extraction and identification

Four millilitre of 0.1 % Sodium chloride was used to dissolve the 0.5 grams of lyophilized culture and 10 ml of methanol was added, then boiled till the methanol gets completely evaporated (in order to rupture the cell membranes). After that 10 ml of chloroform was added and then vortexed for 10 minutes. Then 1 ml of 0.1% NaCl was added and centrifuged for 5 minutes at 25 °C, which resulted in the formation of two layers (chloroform layer and water layer).The chloroform layer at the bottom was trabsferred into round bottom flask and evaporated using the flash evaporator (ROTAVAC). 0.5 ml of the solution of chloroform and methanol in 2:1 ratio was used to dissolve the extract, from which 5 µl was spotted on to the corner of the silica gel TLC plate (TLC silica gel 60 F254 Merck KGaA, 64271 Dermstadt, Germany) and Two dimensional TLC was performed in the solvent system which is described below:

First dimension: Chloroform: methanol: water (30:12:1.5)

Second dimension: Chloroform: methanol: glacial acetic acid: water (35:7:6: 1)

Visualization of lipids was done by spraying 5% ethonolic molybdophosphoric acid on TLC and dried (110 °C) using hot air blower.

2.21.3.1 Ninhydrin reagent (amino group containing lipids)

0.1% ethanolic ninhydrin was sprayed on TLC and dried using hot air gun to visualize amino lipid. The presence of amino group in the lipid leads to formation of pink colouration of the spots.

2.21.3.2 Dragendorff's reagent (Choline containing lipids)

Preparation of the reagent:

Solution A: In 100 ml water/acetic acid (4:1) 1.7g basic bismuth nitrate was dissolved. Solution B: Dissolved 40 g of potassium iodide in water

Mix solutions as follows:

5 ml of solution A + 5 ml of solution B + 20 ml acetic acid + 70 ml water. This was sprayed on plates and orange spots would show the presence of choline containing lipids.

2.21.3.3 Molybdenum blue reagent (Phosphate containing lipid)

Reagent 1 is 25N H_2SO_4 which was used for the preparation of the solutions.

Solution 1; In 1 litre reagent 1, 40.1g of molybdenum trioxide was dissolved.

Solution 2; To 500 ml of reagent 1, 1.78g powdered molybdenum was added and this mixture was boiled gently for 20 minutes. After cooling the solution was transferred and stored in another bottle.

The solutions were mixed in the following ratio;

20 ml of solution 1+20 ml of solution 2+40 ml of double distilled water. Blue coloration of the spots upon spraying on to plates shows positive results.

2.21.3.4 Identification of glycolipids

Visualization of glycolipids was done by spraying 3.2% (w/v) alpha-naphthol in solution of methanol, H_2SO_4 and H_2O (25:3:1.5, vol/vol/vol) and heating the plate at 115 °C. Purple/ pink color spots were considered as glycolipids.

2.21.4 Quinone extraction and identification

From 72 hr grown culture, 1 g of wet pellet was taken in which 2 ml autoclaved H_2O and 10 ml of acetone was added. Then it was sonicated at 50% power for 1hour. The solution was transferred into the 500 ml glass beaker to which 180 ml of acetone was added. To extract maximum amount of quinones, the solution was incubated at 4 °C for overnight. After incubation, the total solution was centrifuged at 900g for 20 minutes at 4 °C. Supernatant was filtered by Whatman filter paper and filtrate was evaporated under vacuum. 90 ml of autoclaved double distilled water and 180 ml of n-hexane was added in this. Total solution was properly mixed for 20 minutes in shaking incubator at 100 rpm at room temperature. Total solution was kept outside for 30 minutes which formed two layers. Water layer was gently removed. Na_2SO_4 was used to remove residual water. The n-hexane layer was evaporated under vacuum and 1 ml. of absolute ethanol was used to dissolve the final quinone extract.

2.22 Genotypic and phylogenetic characterization methods

2.22.1 Genomic DNA isolation

1 gram of wet pellet was taken in 50 ml centrifuge tube

⇩

Washed twice with Milli-Q water

⇩

10 ml buffer (SET buffer; 25 mM EDTA, pH 8.0: 20mM Tris- HCl, pH 8.0: 75mM NaCl) was used to re-suspend the cell pellet

⇩

To the above cell suspension proteinase K (200 µl of 50 mg/ml) and SDS (1ml of 50% w/v) was added

⇩

Incubated for 1 hour at 55 °C in water bath

⇩

Incubated at 37 °C for half an hour after adding 2 ml of 5 M NaCl and equal volume of tris buffer saturated phenol: chloroform: isoamyl alcohol (25: 24: 1)

⇩

Centrifuged the above mixture at 7,000 for 15 minute, at 37 °C.

⇩

To the aqueous phase sodium acetate (0.1 volume containing pH 4.8) and chilled absolute ethanol (1 volume) were added by following gentle extraction and then 30 min incubation at room temperature.

⇩

The above mixture was centrifuged for 5 min at 10K rpm at 4 °C. Washing of cell pellet was done by using 70% ethanol for 5 min at 10K rpm at 4 °C. After air dry, pellet was dissolved in TE buffer and store at 4 °C.

2.22.2 16S rRNA gene amplification (Colony PCR), sequencing

A single, well separated colony was dissolved in 50 µl of autoclaved milli Q water taken in 200 µl centrifuge tube and kept for denaturation in PCR machine (BIO-RAD MJ Mini, personal thermal cycler) for 15 min at 94 °C. Two set of primers (F8 or F27 with R1492/ F8 or F27 with R1525 numbering as per *Escherichia coli* 16S

rRNA numbering system [Lane et al., 1985; Brosius et al., 1978; Liesack et al., 1991]) were used for amplifying the 16S rRNA gene.

Primers sequences are as follows:

S No	Primer	Name	Sequence 5'to 3'
1	Forward	F8	5´- AGAGTTTGATCCTGGCTCAG -3´
2	Forward	F27	5´- GTTTGATCCTGGCTCAG- 3´
3	Reverse	R1492	5´-GGTTACCTTGTTACGACT-3´
4	Reverse	R1525	5´-AGAAAGGAGGTGATCCAGCC-3´

For PCR amplification TAKARA master mix (EmeraldAmp® GT PCR Master Mix) was used instead of individual components. PCR Reaction mixture prepared as follows;

Component	Volume (µl)
Milli-Q water	16
MasterMix	25
Forward Primer	2
Reverse Primer	2
Template DNA (from denatured colony, 50 µl)	5

PCR conditions for 16S rRNA gene amplification were as follows;

S No	Step	Temp (°C)	Time
1	Initial denaturation	94	10 minutes
2	Denaturation	94	1 minute
3	Annealing	50-55	0.5 minute
4	Elongation	72	1.5 minutes
	Number of cycles: 32		
5	Final elongation	72	10 minutes in last cycle

1.0% agarose gel was prepared in TAE buffer to run the amplified product along with DNA marker at 80 voltage for 40 min. Ethidium bromide staining was used for visualization and the amplified products were observed using the BIO-RAD gel

documentation system. Desired amplified products were outsourced to Agrigenome Labs Pvt. Ltd. for sequencing where the 16S rRNA gene sequencing was carried out in an ABI 3730 XL DNA Analyzer automatic sequencer using the BigDye1 Terminator v.3.1 Cycle Sequencing Kit. The sequences obtained from different primers were assembled using DNA Lasergene SeqMan Pro software. The assembled sequence similarity with existing bacteria were found by BLAST search using the EzBioCloud database (Yoon et al., 2017). For multiple sequence alignment MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/), for pairwise sequence alignment LALIGN algorithms (https://www.ebi.ac.uk/Tools/psa/lalign/) was used.

2.22.3 Phylogenetic analysis

Phylogenetic analysis was performed with MEGA6/MEGA7 software (Tamura et al., 2013; Kumar et al., 2016). For 16S rRNA gene-based phylogenetic analysis, sequences of all strains were extracted from the NCBI database. For nucleotide based trees (16S rRNA gene and *rpoB* gene) distances were calculated by using the Kimura2-parameter (Kimura, 1980) in a pairwise deletion procedure and Poisson model (Zuckerkandl and Pauling, 1965) for the protein sequences of PufX, PufL,M and photosynthetic gene cluster (PGC) based phylogenetic trees. Neighbor-Joining (NJ), Maximum Likelihood (ML) and Minimum Evolution (ME) methods in the MEGA6/MEGA7 software were used to construct phylogenetic trees. To assess the reliability of phylogenetic tree bootstrap was set to 1000.

2.22.4 G+C content analysis

The DNA samples including 4mg each of genomic DNA and standard DNA (HiMedia) were degraded into individual nitrogen bases using 100 µl of $HClO_4$ (perchloric acid) in a glass tube in water bath for 60 minutes at 100 °C. Using a glass rod, hydrolysed sample was mixed to get a homogenized black char. Milli-Q water was added to this to make the final volume 0.5ml and then centrifuged. The supernatant was taken into another tube and filtered using 0.2 µm cellulose nitrate filters.

2.22.4.1 Preparation of reference DNA bases and solvent preparation

Purines (adenine, guanine) and pyrimidine (cytosine, thymine) of HiMedia made, were dissolved in HCl to get 1mM concentration. The solvent for chromatography was prepared by adding 750 ml Milli-Q water and 40 ml of 0.5M TEAP (triethylamine phosphate) of pH of 5.1, then the final volume was made to 1 litre with methanol (HPLC grade). The solvent was filter sterilized and at 1.0 ml/min flow rate at room temperature. The preparation of 0.5 M TEAP was done in water and phosphoric acid was used to adjust the pH to 5.1.

2.22.4.2 HPLC Analysis

Twenty microliters of each base were injected and noted the retention time. Then the acid hydrolysed DNA sample was injected and by comparing with the standards detection and concentrations, the G+C mol % was calculated using the formula:

$$\textbf{Mol\% G+C} = \textbf{[}(G+C)/(A+T+G+C)\textbf{]}\ \textbf{X}\ 100$$

2.22.5 DNA relatedness calculating tools

2.22.5.1 DNA-DNA hybridization

Membrane filter technique was employed to perform DNA-DNA hybridization as reported by Tourova and Antonov (1987), which has three major steps:

A. Immobilization of DNA.

B. Labelling of DNA with radioactive material.

C. Hybridization of immobilized DNA with radiolabeled DNA.

A. Immobilization of DNA

Purified DNA (10 µg) was taken in a 15 ml centrifuge tube. 20X SSC was added to the tube in such a way that the final concentration of SSC should be 6X. The mixture was placed in boiling water bath for 10 min to denature the DNA strands. The centrifuge tube was then transferred to ice in-order avoid the renaturation of strands. The denatured DNA was immobilized on the nylon membrane (Amersham Hybond-N-GE Healthcare) using a dot blot apparatus. 0.5N NaOH was used to wash the wells, after which the membrane was dried at 80 °C for 2 hours under vacuum.

B. Labelling of DNA with radioactive material using Nick translation

In Nick translation the DNA nucleotides were replaced by ^{32}P-labelled nucleotides which will generate DNA with radioactive nucleotides with the help of DNA polymerase I, which has capacity of both 3' OH Polymerase activity (addition of nucleotides) and 5' to 3' exonuclease activity (deletion of nucleotides). The Nick translation was carried out in 1.5 ml micro centrifuge, ingredients are given in Table 2.2.

Table 2.2 The reaction mixture ingredients used for nick translation.

DNA	5 µl (200ng)
dATP	5 µl
dTTP	5 µl
dGTP	5 µl
α-P32 dCTP	5 µl
Milli-Q H_2O	70 µl

The reaction mixture was incubated at 15 °C for 2 hours and reaction was stopped by adding the 8 µl of EDTA (0.25 M). The reaction mixture was then transferred to the 15ml falcon tube containing the 6X SSC buffer and denature the radiolabelled DNA (by boiling) and transfer immediately to avoid the renaturation.

C. Hybridization of immobilized DNA with radiolabelled DNA.

The dried nitrocellulose membrane paper was soaked for 60 min at 55-60 °C in the prehybridization buffer (7 % SDS containing 0.5 M phosphate buffer having pH of 7.2). Meanwhile the purified probe was dissolved in the prehybridization buffer. Immobilized DNA and probe hybridization were done at 55- 60 °C for 16 h. The membrane was washed with 0.5 X SSC containing 0.1% SDS for 10 min at room temperature and 0.1X SSC containing 0.5% SDS for 20 min at 50 °C. The membrane filter was dried and exposed to phosphor screen (Amersham Biosciences) for 4 hours. The screen scanning and quantification done by phosphoimager (TYPHOON 9400). The DNA-DNA reassociation values were calculated using the formula;

$$\% \text{ Hybridization} = \frac{\text{Counts obtained from heterologous hybridization}}{\text{Counts obtained from homologous hybridization}} \times 100$$

2.22.6 Digital/ *In-silico* DNA-DNA hybridization (*d*DDH)

The advancement in the genome sequencing technology replaces the error prone conventional DNA-DNA hybridization with *in-silico* DNA-DNA hybridization. *In-silico* DNA-DNA hybridization values between the organisms of this study were obtained from the Genome-to-Genome Distance Calculator 2.1 (https://ggdc.dsmz.de/ggdc.php#; Meier-Kolthoff et al., 2014).

2.22.7 Average Nucleotide Identity (ANI)

ANI score between the organisms were calculated from Kostas lab ANI calculator (http://enve-omics.ce.gatech.edu/ani/; Rodriguez-R and Konstantinidis, 2014).

2.22.7.1 Orthologous Average Nucleotide Identity (OrthoANIu)

OrthoANI score between the organisms using the USEARCH were calculated from Chun lab's online Average Nucleotide Identity calculator (https://www.ezbiocloud.net/tools/ani; Yoon et al., 2017).

2.22.8 Average Amino acid Identity (AAI)

AAI values between the organisms in this study were calculated from Kostas lab AAI calculator (http://enve-omics.ce.gatech.edu/aai/; Rodriguez-R and Konstantinidis, 2014).

2.22.9 Percentage Of Conserved Proteins (POCP)

With the help of BLASTP program query genome protein sequences were aligned with the reference genome to get the conserved proteins between the two organisms. To consider the conserved proteins of query genome

A. They should have a BLAST match with an E value of less than 1e−5,

B. 40% or more identity

C. It should align more than 50%.

The number of conserved proteins was calculated by performing the BLASTP and by using each genome as query genome.

Between the two genomes POCP value was calculated using the formula

$[(C1 + C2)/(T1 + T2)] \times 100\%$,

Where $C1$, $C2$ represent the number of conserved proteins, $T1$ and $T2$ represent the number of total proteins of the two genomes being compared. The POCP values can vary between 0 to 100%.

2.23 Phylogenomics

2.23.1 Construction of phylogenomic tree using UBCG (Up-to-date bacterial core gene) pipeline

The workflow for construction of UBCG tree was given in the Fig. 2.2

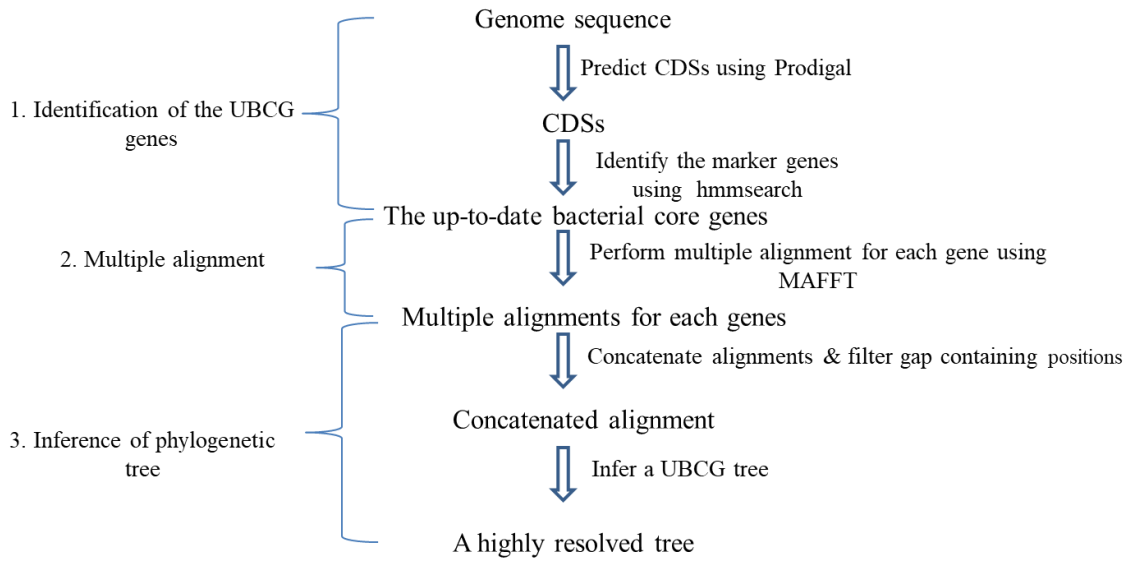


Fig. 2.2 The flowchart of phylogenetic tree reconstruction using the UBCG pipeline is adopted from Na et al. (2018).

2.23.2 Protein based phylogenomic tree

The phylogenomic tree was also deduced from the genome translated amino acids using the automated pipeline of the Patric online server (https://patricbrc.org). In brief, the Patric server begins with amino acid sequence files for each genome. Homologous proteins were identified in two rounds. BLAST was used in first round in which the genome of each distinct species is searched against other genomes and an MCL (Markov Cluster) algorithm was used to cluster the top scoring hits, which are initial seed sets for the homology groups. Alignment of seed sets was carried out by MUSCLE, and hmmbuild was used to build Hidden Markov Models (HMMs). The phylogenomic tree was generated with FastTree and RAxML from the concatenated alignment. Instead of bootstraps, trees are built from random samples of 50% of the homology groups used for the main tree, in a process referred to as gene-wise jackknifing. 100 of these 50% gene-wise jackknife trees are made using FastTree/RAxML, and the support values shown indicate the number of times a particular branch was observed in the support trees.

2.24 Pan-genome

Bacterial Pan- Genome Analysis (BPGA) - an ultra-fast pan-genome analysis pipeline (Chaudhari et al., 2016), with default criteria was used to perform pan-genome. In first step BPGA modifies the input files by inserting genome ID into the sequence headers which is called as preparation step, then clustering of genes into orthologous clusters using USEARCH, preparation of binary matrix based on the presence or absence of genes from the individual strains, Calculation of shared genes after stepwise addition of each individual genome. This trend can be plotted as core or pan-genome profile curves. Finally, BPGA script provides genome wise core,

accessory, unique and exclusively absent gene counts, which gives idea about contribution of each strain to the pan-genome.

Genomic information used in this study

The genome sequences of type strains of *Rba. blasticus*, *Rba. veldkampii* and *Cereibacter changlensis* were shared by Prof. Meyer Terrance, prior to their release to the NCBI database. Type strains of the genus *Rhodobacter,* and other genome sequences used in the study were downloaded from the NCBI database (Table 2.3)

Table 2.3 The organisms used in this study, isolation sources and genome accession numbers

Organisms	Source of Isolation	NCBI accession number
Rhodobacter capsulatus DSM 1710^T	Sewage plants, eutrophic ponds	QKZO00000000
Rhodobacter viridis JA737^T	Mud of a stream	QJTK00000000
Rhodobacter maris JA276^T	Marine sediment	OBMT00000000
Rhodobacter aestuarii JA296^T	Estuarine environment	FTOG00000000
Rhodobacter sphaeroides 2.4.1^T	Sewage plants, eutrophic ponds	CP030271 and CP030272
Rhodobacter johrii JA192^T	Rhizosphere soil	MABH00000000
Rhodobacter megalophilus JA194^T	Soil, Himalayas	FZOV00000000
Rhodobacter azotoformans KA25^T	Sludge for waste water treatment	QAOT00000000
Rhodobacter ovatus JA234^T	Polluted pond	OAOQ00000000
Rhodobacter vinaykumarii JA123^T	Seawater	OBMN00000000
Rhodobacter veldkampii ATCC 35703^T	Freshwater	PZKF00000000
Rhodobacter blasticus DSM 2131T	Eutrophic pond	PZKE00000000
Rhodobacter sediminicola JA983^T	Freshwater sediment	VDEK00000000
Rhodobacter thermarum YIM 73036^T	Sediment sample of a hot spring	QMJY00000000
Rhodobacter flagellatus SYSU G3088^T	Whitish streamers sediment mixed	VMDU00000000

	slurry	
Cereibacter changlensis JA139^T	Snow sample	PZKG00000000
Defluviimonas denitrificans DSM 18921^T	Marine aquaculture	PVEP00000000
Falsirhodobacter sp. alg1	Brown algae	BBJC00000000
Haematobacter missouriensis CCUG 52307^T	Septicemic patient	JFGS00000000
Paenirhodobacter enshiensis DW2-9^T	Soil	JFZB00000000
Pseudorhodobacter ferrugineus DSM 5888^T	Seawater of the Baltic Sea	ATVN00000000
Thioclava pacifica DSM 10166^T	Near-shore sulfidic hydrothermal area	AUND00000000
Gemmobacter aquatilis DSM 3857^T	Fresh water	FOCE00000000
Pararhodobacter aggregans DSM 18938^T	Marine aquaculture	QBKF00000000
Rhodobaca barguzinensis alga-05^T	Sediments of a saline	CP024899
Roseicitreum antarcticum CGMCC 1.8894^T	Sandy intertidal sediment	FNOM00000000
Roseinatronobacter thiooxidans DSM 13087^T	Soda lakes	MEHT00000000
Rhodovulum sulfidophilum DSM 1374^T	Sea sediment	CP015418
Albimonas donghaensis DSM 17890^T	Seawater	FNMZ00000000
Paracoccus denitrificans DSM 413^T	Soil	FNEA00000000
Roseobacter litoralis Och 149^T	Seaweed	CP002623
Escherichia coli ATCC 11775^T	Human, urine, cystitis	JMST00000000
Pseudomonas aeruginosa DSM 50071^T	Soil and water	JYLC00000000
Sinorhodobacter ferrireducens CCTCC AB2012026^T	Microbial fuel cell	SAVB00000000
Roseibaca ekhonensis CECT 7235^T	Lake water	UIHC00000000

Results

3. Results

3.1 Enrichment, purification and typing

Out of the 72 samples analyzed, 48 samples showed appearance of yellowish brown/ brownish color in the enrichment bottles or reddish brown colonies on agar plate (Fig. 3.1). These are the characteristic color of the members of genus *Rhodobacter*. These were purified by repeated streaking on agar plates/ agar slants and were preserved in glycerol at -20 °C.

Based on phenotypic characteristics [phototrophically grown cultures color, presence of bacteriochlorophyll (BChl *a*/BChl *b*), cell shape, motility, mode of reproduction], cultures were sent for identification of bacterial strains. Out of 48, 32 (Table 3.1) representative strains 16S rRNA gene amplification and sequencing were done for which the sequence identity were determined by BLAST analysis using EzBioCloud.

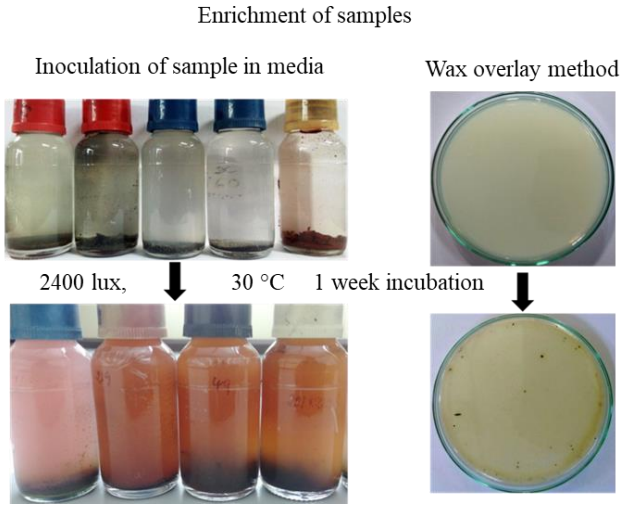


Fig. 3.1 Image of enrichment bottles/ wax overlay plate before and after incubation, appearance of yellowish brown/reddish brown colonies on wax overlay plate.

Table 3.1 Sampling sites of newly isolated strains and their 16S rRNA gene identity with existing species.

S. No	Sample no.	Place	GPS	Nature of Sample	Strain isolated	EzBioCloud based 16S rRNA sequence identity	EMBL accession no.
1	1	**Bhavnagar**	**21° 700'N 72° 228'E**	Decaying algal mat + Black sediment	*Rhodobacter* sp. JA1033	100% with *Rba. sphaeroides* ATH 2.4.1^{T}	LR739226
2	5			Algal mat with black soil	*Rhodobacter* sp. JA1040	99.9% with *Rba. johrii* JA192^{T}	LR739231
3	**6**			**Water with sediment**	***Rhodobacter* sp. JA983^{T}**	**98.9% with *Rba. johrii* JA192^{T}**	**LR596790**
4	10	Ghogha beach	21°77'N 72° 06'E	Subsurface soil	*Rhodobacter* sp. JA1034	99.6% with *Rba. johrii* JA192^{T}	LR739229
5	13			Brown mat on soil	*Rhodobacter* sp. JA1035	99.8% with *Rba. sphaeroides* ATH 2.4.1^{T}	LR739230
6	14			Purple layer on mud	*Rhodobacter* sp. JA1036	100% with *Rba. sphaeroides* ATH 2.4.1^{T}	LR739232
7	21	Nari salt pan	21° 797'N 72° 069'E	Thick layer of brown	*Rhodobacter* sp. JA1037	99.9% with *Rba. johrii* JA192^{T}	LR739239
8	26			Brown layer on sediment	*Rhodobacter* sp. JA1038	99.7% with *Rba. johrii* JA192^{T}	LR739240
9	30	Along ship yard	21° 442'N 72° 225'E	Brown layer on soil	*Rhodobacter* sp. JA1041	99.4% with *Rba. johrii* JA192^{T}	LR739238
10	36	Bhavnagar to kambhat	21° 940'N 72° 077'E	Water + sediment	*Rhodobacter* sp. JA1042	99.1% with *Rba. johrii* JA192^{T}	LR740730
11	**42**	**Mahemdavad**	**22° 474'N 72° 431'E**	**Fresh water pond filled with *Azolla***	***Rhodobacter* sp. JA912^{T}**	**99.2% with *Rhodobacter viridis* JA737^{T}**	**LN810641**

12					*Rhodobacter* sp, JA913	99.2% with *Rhodobacter viridis* JA737^T	LN810642
13	47	Khambhat	21° 475'N 72° 431'E	Wheat field rhizosphere	*Rhodobacter* sp. JA914	100% with *Rhodobacter megalophilus* JA194^T	LN810643
14					*Rhodobacter* sp. JA915	100% with *Rhodobacter megalophilus* JA194^T	LN810644
15	49	Sabarmati river	22° 381'N 72° 233'E	Brown layer on water biofilm	*Rhodobacter* sp. JA1039	99.4% with *Rba. sphaeroides* ATH 2.4.1^T	LR740731
16	52	Gopnath	21° 210'N 72° 109'E	Brown layer on water biofilm	*Rhodobacter* sp. JA1043	99.8% with *Rhodobacter johrii* JA192^T	LR740732
17					***Rhodobacter* sp. JA916^T**	**98.4% with *Rhodobacter johrii* JA192^T**	**LN810645**
18	**64**	**Jafarabad**	**20° 92' N 71° 41' E**	**Alkaline brown pond sediment**	*Rhodobacter* sp. JA917	98.41% with *Rhodobacter johrii* JA192^T	LN810646
19					*Rhodobacter* sp. JA918	98.41% with *Rhodobacter johrii* JA192^T	LN810647
20					*Rhodobacter* sp. JA919	98.41% with *Rhodobacter johrii* JA192^T	LN810645
21	77	Fudam bird sanctuary	20° 71' N 70° 96' E	Algal mat + sediment	*Rhodobacter* sp. JA1045	99.9% with *Rba. sphaeroides* ATH 2.4.1^T	LR740733
22	95	Porbander	21° 66' N 69° 6' E	Sediment	*Rhodobacter* sp. 1044	99.1% with *Rhodobacter johrii* JA192^T	LR740751
23	113	Kachigadi	22° 331' N 68° 951' E	Black anaerobic soil	*Rhodobacter* sp. JA1051	99.1% with *Rba. sphaeroides* ATH 2.4.1^T	LR740735
24	121			Soil	*Rhodobacter* sp. JA1052	100% with *Rba. sphaeroides* ATH 2.4.1^T	LR740736
25	136	Sikka	N 22°36' E 69°829'	Brown layer below rock	*Rhodobacter* sp JA1053	99.8% with *Rhodobacter johrii* JA192^T	LR740749

26	GJS19	Nandanvan	N 22° 26' E 69° 06'	Aquatic oligotrophic plant	*Rhodobacter* sp JA1044	100% with *Rba. sphaeroides* ATH 2.4.1^T	LR740751
27	GJS32	Next to porbandar	21°41'218" N 69° 33' 612"E	Biofilm on Rock	*Rhodobacter* sp. JA1042	99.5% with *Rhodobacter johrii* JA192^T	LR740730
28	GJS133	Jogad	23° 34' N 72° 46' E	Yellow color Sponge	*Rhodobacter* sp. JA886^T	99.6% with *Rhodobacter vinaykumarii* JA123^T	LT799384
29	GJS223	White Rann way	21° 41' 218 '' N 69° 33' 612' E	Sediment of brown pond	*Rhodobacter* sp. JA1054	99.7% with *Rba. sphaeroides* ATH 2.4.1^T	LR740750
30	GJS15	Kandla port	23°81' N 71°47' E	Sediment	*Rhodobacter* sp. 1048	99.2% with *Rba. johrii* JA192^T	LR740752
31	GJS18			Brown water + sediment	*Rhodobacter* sp. 1049	99.4% with *Rba. alkalitolerans* JA192^T	LR740754
32	GJS23			Brown pond sediment	*Rhodobacter* sp. 1050	99.92% with *Rba. johrii* JA192^T	LR739228
			Members isolated other than *Rhodobacter*				
33	SH8	Umiam lake, Shillong	25° 68' 18" N 91° 92' 53" E	Rock with biofilm	*Rhodomicrobium* sp. JA980	99.6% with *Rhodomicrobium vannielii* ATCC17100^T	LT934242
34					*Rhodomicrobium* sp. JA979	99.6% with *Rhodomicrobium vannielii* ATCC17100^T	LT934238
35	GJ136	Jogad, GJ	23° 34' N 72° 46' E	Algal biofim	*Rhodoplanes* sp. JA981	99.2% with *Rhodoplanes pokkalisoli*	LR740769

Based on the 16S rRNA gene sequence identity and preliminary studies (yellowishbrown/ brownish red color, rod to oval shape cells, binary fission mode of cell division), out of thirty two strains, four strains belongs to genus *Rhodobacter* (Bold) and a strain of *Rhodomicrobium* (non-*Rhodobacter* member) genus was also characterized using the polyphasic taxonomic approach.

3.2 Polyphasic characterization

3.2.1 Characterization of strain JA912^T and strain JA913

3.2.1.1 Habitat, colony and cell morphology and photosynthetic membrane architecture

Strains JA912^T and JA913 were isolated from *Azolla filiculoides* of a fresh water pond (Fig. 3.2A) in Gujarat, India (GPS position: 72º43' E, 22º47' N; sample pH 6.0 and salinity 0.8%). Both strains formed round shaped, yellowish brown color colonies when it was grown anaerobically (Fig. 3.2B,C). Cells of strain JA912^T and JA913^T were rod to oval shaped (0.8-1.5 x 1.0-3.0 μm; Fig. 3.2D), multiplied by binary fission and were motile by means of single polar flagellum (Fig.3.2E), Transmission electron microphotographs of ultrathin sections of the strain JA912^T revealed the presence of vesicular type of internal membrane architecture (Fig. 3.2F)

3.2.1.2 Pigment and quinone composition

The *in vivo* absorption maxima at 476, 509, 590, 800, and 851 nm (Fig. 3.3) indicating the presence of bacteriochlorophyll a and carotenoids. HPLC analysis indicated the presence of spheroidene as the major carotenoid and ubiquinone-10 (Q10) as predominant quinone system in both the strains.

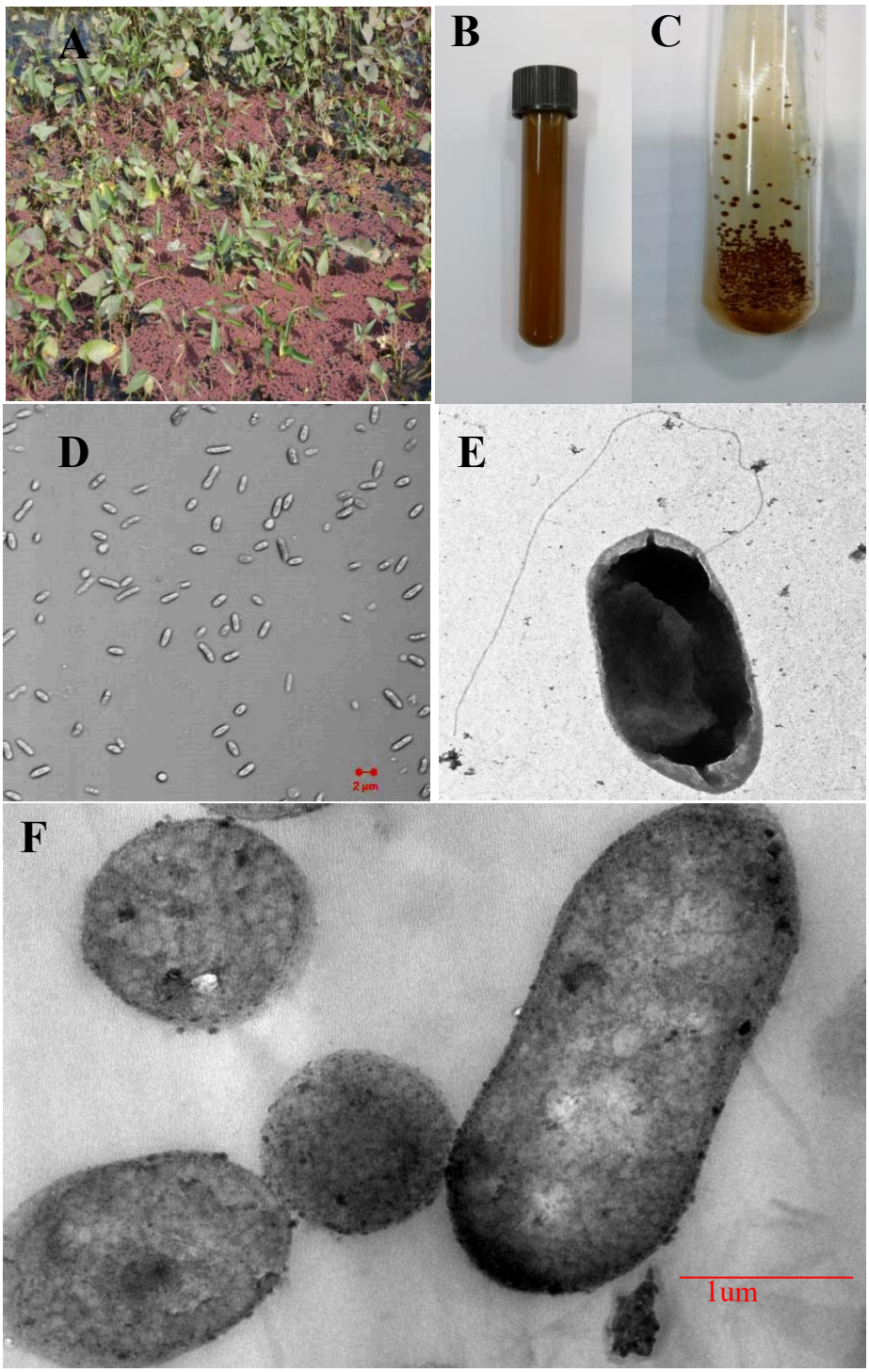


Fig. 3.2 Image showing the characteristics of strain JA912^{T}; A, sampling site; B, Image showing the color of phototrophically grown culture; C, Image showing the color and shape of the colony; D, Confocal micrograph of strain JA912^{T}; E, Electron micrograph of negatively stained cells; F, electron micrograph of ultrathin sections showing the vesicular architecture of ICM

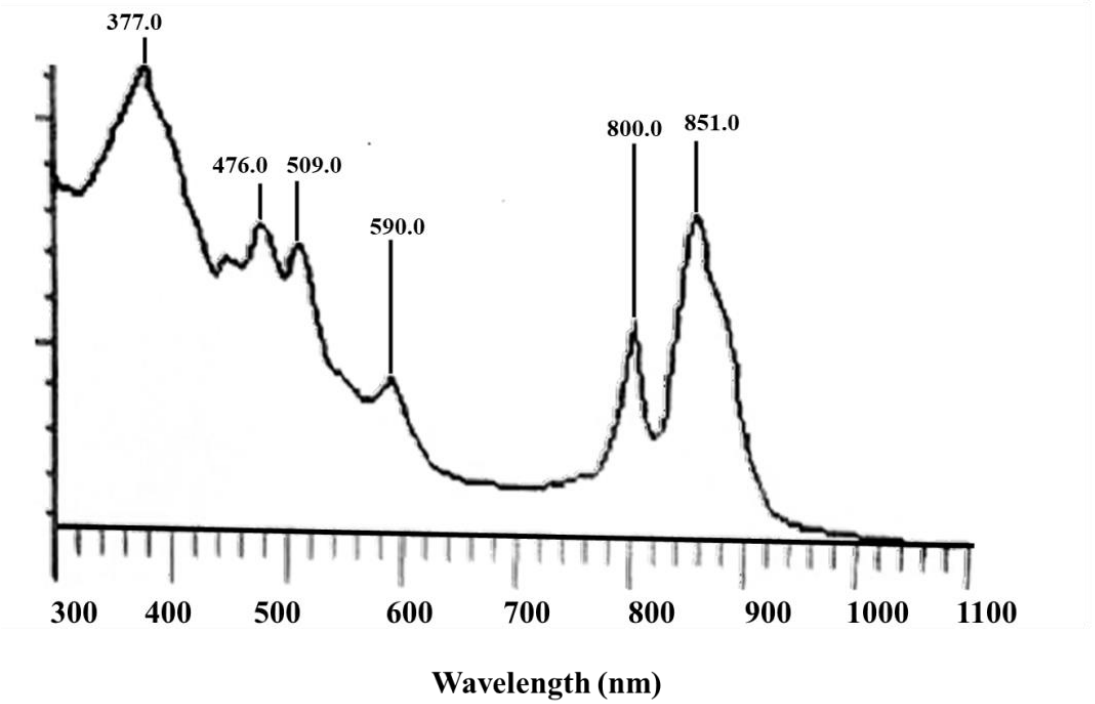


Fig. 3.3 Whole-cell absorption spectrum of strain JA912^{T}

3.2.1.3 FAME analysis

Both strains have $C_{18:1}\omega7c/C_{18:1}\omega6c$ as the major (>10%) fatty acid, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{10:0}3OH$, $C_{18:1}\omega5c$, $C_{18:0}3OH$, $C_{16:0}$ and $C_{18:0}$ as minor quantity (>1 but <10%) and the fatty acid. The differences of between strain JA912^{T} with their phylogenetic neighbours are given in Table 3.2.

Table 3.2 Differential characteristics of strain JA912^T with the closely related species of the genus *Rhodobacter*

Characteristics	1	2	3	4
Color of cell suspension	YB	RB	GR	YB
Cell shape	O-R	R	O-R	O-R
Chain formation of cells	-	-	-	+
Cell size(µm)	1.0-1.5 x 1.0-3.0	0.5-0.7 x 2.1-4.0	0.5–1.2 x 2.0–2.5	0.5-1.2 x 2.0-2.5
Major carotenoid	SE	SE	NS	SE
Photolithoautotrophy	-	+	+	+
Slime production	-	+	-	+
NaCl requirement	-†	-§	-	-
Growth at pH range (optimum)	6-8 (7.0)	6.0-9.0 (6.5-8.0)	6.0-9.0 (6.5-8.0)	6.5-8.0 (6.5-7.5)
Growth at temperature °c (range)	25-35	12-45	30	20-30
Vitamin requirement	b,t	Nr	T	b,t
Oxidase	+	+	+	+
Hydrogen	-	+	+	+
Sulfide	-	+	-	+
Thiosulfate	-	+	+	+
Fumarate	+	+	-	-
Succinate	+	+	-	
Glucose	+	+	+	-
Fructose	+	+	+	-
Sucrose	+	+	+	-
Aspartate	+	+	-	-
Glutamate	-	+	-	+
Mannitol	+	+	+	+/-
Glycerol	+	+	-	-
Citrate	-	-	-	+/-
Acetate	+	+	-	+
G+C content	64.6	70.6	66.4	68.8
Fatty acid composition (%)				
$C_{16:0}$	6.0	4.9	2.0	3.9
$C_{17:0}$	0.2	1.1	0.4	1.1
$C_{18:0}$	3.8	6.7	-	7.8
$C_{10:0}$3OH	1.4	-	2.2	2.1
$C_{18:0}$3OH	1.7	-	1.1	2.2
$C_{16:1}\omega7c/C_{16:1}\omega6c$	10.6	6.1	11.2	5.4
$C_{18:1}\omega5c$	1.4	2.4	16.1	-
$C_{18:1}\omega7c/C_{18:1}\omega6c$	68.1	66.7	61.2	67.3

species: 1, strain JA912^T; 2, *Rba. sediminis* N1^T ; 3, *Rba. viridis* JA737^T; and 4, *Rba. capsulatus* LMG 2962^T.

All strains are motile, divide by binary fission, have vesicles as intracytoplasmic membrane *(*ICM*)* architecture, Q10 quinone system and polar lipids phosphatidylglycerol, phosphatidylcholin, phosphatidylethanolamine, unidentified lipids. Pyruvate and malate were utilized by all the strains. All strains are catalase, tryptophanase (indole production) positive. Benzoate, tartrate and ethanol were not utilized by any of the strains. None of the strains are positive for lipases, urease, casease, citrate permease (citrate), nitrate reductase, acetoin dehydrogenase (VP test), cysteinase (H_2S production). O-R, oval to rod, YB, yellowish brown, GR, green; RB, reddish brown SE, spheroidene; NS, neurosporene; b, biotin; t, thiamine; paba, p-aminobenzoicacid; nr, not required; +, present/ utilized; -, absent/not utilized; †NaCl not required for growth, but can grow up to 2%; §NaCl not required for growth, but can grow up to 3%.

3.2.1.4 16S rRNA gene sequence similarities and phylogenetic affiliation

The BLAST (EzBioCloud) search analysis indicated that both strains shared highest 16S rRNA gene sequence identity with the type strains of the genus *Rhodobacter*. Strain JA912^T have highest sequence similarity with *Rba. viridis* JA737^T (99.63%), *Rba. sediminis* N1^T (99.27%), *Rba. capsulatus* ATCC 11166^T (98.77%) and less than 97% with other members of the genus *Rhodobacter*. 16S rRNA gene sequence similarity of strain JA912^T with JA913^T was 100%. A phylogenetic tree (NJ) constructed by MEGA6 software revealed (Fig. 3.4) that strain JA912^T (including strain JA913) formed distinct sub-clade with *Rba. sediminis* N1^T, *Rba. viridis* JA737^T, and *Rba. capsulatus* ATCC 11166^T.

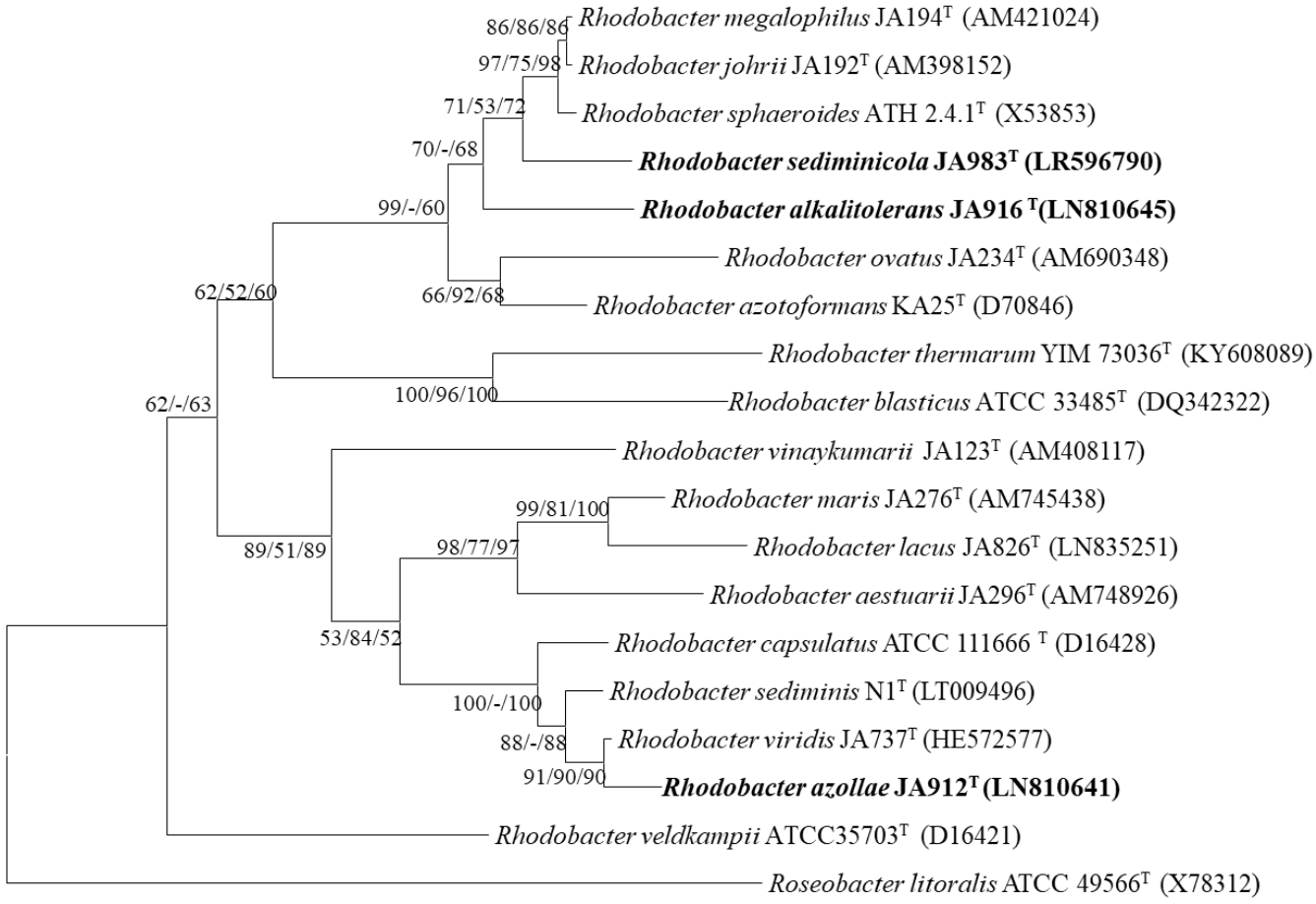


Fig. 3.4 16S rRNA gene sequences based phylogenetic tree showing the phylogenetic relationship of strains JA912^T, JA916^T and JA983^T with the members of genus *Rhodobacter*

3.2.1.5 Physiological and Biochemical analysis

Both strains were able to grow photoorganoheterotrophically and chemoorganoheterotrophically. but couldnot grow chemolithoautotrophically, fermentatively photolithoautotrophically. NaCl was not obligatory for growth, however, they could tolerate up to 1-2%. Both strains required pH 7.0 for optimum growth but tolerated from 6.0 to 8.0. Both strains are mesophiles, growing optimally at 30ºC (range 25-35ºC).

Both the strains (JA912^{T} and JA913) could grow with pyruvate, fructose, lactate, malate, succinate, fumarate, glucose, sucrose and aspartate. Those which could not be utilized by both include; benzoate, ethanol, methanol, propanol and glutamate (Table 3.2). Both strains grew well with ammonium chloride, aspartate and glutamate as nitrogen sources while, nitrite and nitrate did not support growth of either strains. Strains showed diazotrophic growth and nitrogenase activity. Both strains utilized sulfide, sulfate, and thiosulfate as sulfur sources, while methionine, cysteine, elemental sulfur did not support growth. Both strains required vitamins like biotin and thiamine for growth. The *in vivo* absorption maxima at 476-493, 509-512, 590, 800-803, and 851-860 nm (Fig. 3.3) indicating the presence of bacteriochlorophyll *a* and carotenoids

3.2.1.6 Polarlipid profile

Both strains have phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC) and aminolipids (AL1, AL2), an unidentified lipids (L1-L7), two unidentified phosopholipids (PL1,2; Fig. 3.5).

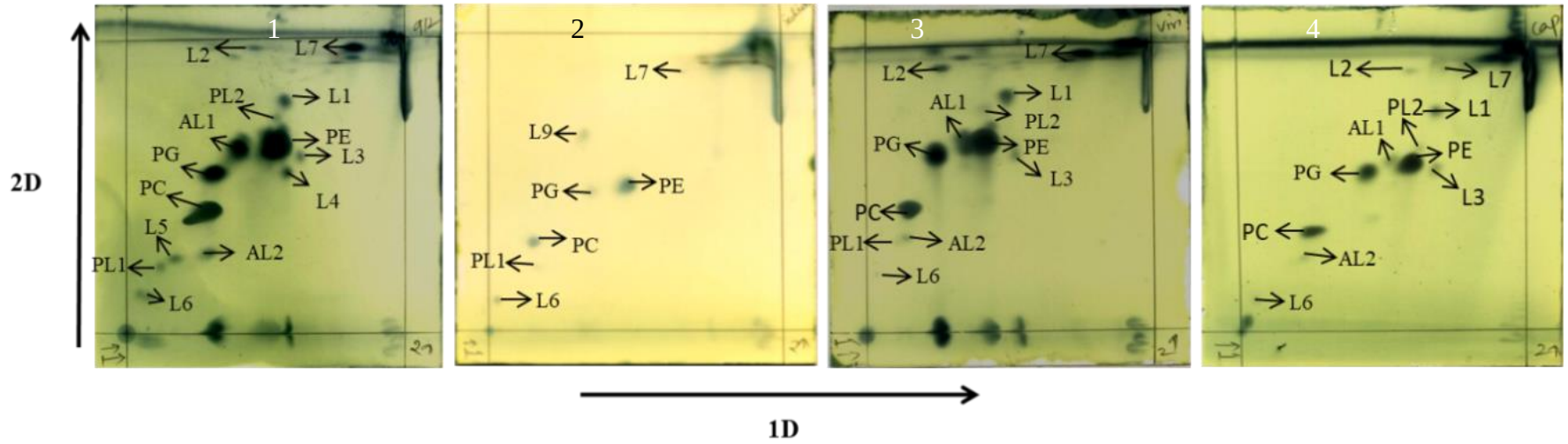


Fig. 3.5 Two-dimensional thin-layer chromatogram showing the polar lipid profile of strain 1, JA912^{T} ; 2, *Rba. sediminis* N1^{T} ; 3, *Rba. viridis* JA737^{T} and 4,)*; Rba. capsulatus* LMG 2962^{T}. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PL1,2, uncharacterized phospholipids; AL1,2, uncharacterized aminolipids; L1-9 unidentified lipids.

3.2.1.7 DNA-DNA hybridization

For DNA-DNA hybridization studies, strain JA912^T DNA was radioactively labeled, the levels of DNA–DNA re-association with type strains of *Rba. sediminis* N1^T, *Rba. viridis* JA737^T, *Rba. capsulatus* ATCC 11166^T were 44±3, 39±1, and 30±4% respectively (Fig. 3.6). However, when the type strains of *Rba. sediminis* N1^T, *Rba. viridis* JA737^T, *Rba. capsulatus* LMG 2962^T (=ATCC 11166^T) were labeled and used for DNA–DNA hybridization with JA912^T in the reciprocal reaction, the re-association values were 45±1, 37±2 and 29±1%, respectively. Between the both strains 96% relatedness observed.

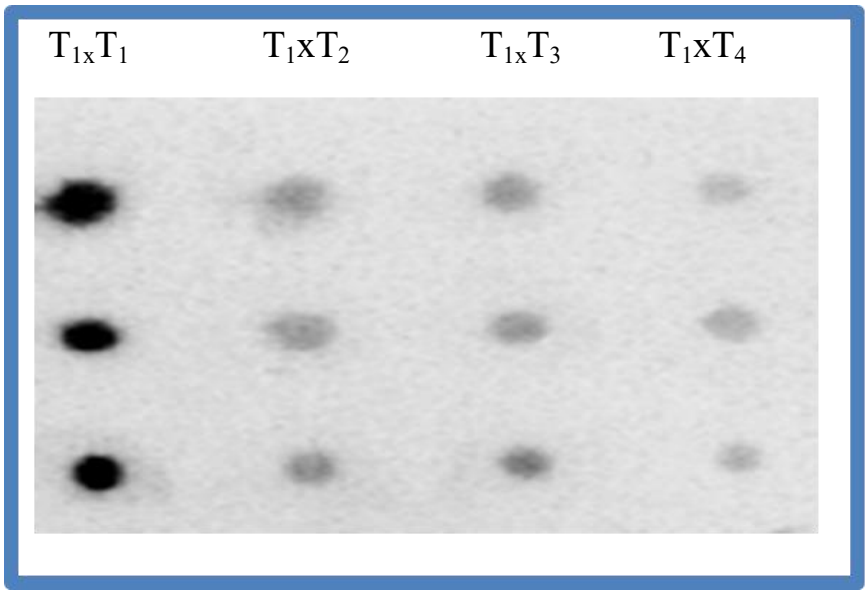


Fig. 3.6 Dot-blot hybridization analysis of strain JA912^T (T_1), *Rba. sediminis* N1^T (T_2); *Rba. viridis* JA737^T (T_3); *Rba. capsulatus* LMG 2962^T (T_4).

3.2.2 Characterization of strain JA916^T

3.2.2.1 Habitat, colony and cell morphology, photosynthetic membranes

Sediment sample collected from a brown colored pond (Fig. 3.7A) in Gujarat, India (GPS position: 20°55'39"N 71°24'43" E) had a pH of 9.0-9.5 and salinity of 0.5%. Color of phototrophically grown culture was yellowish brown/brownish red (Fig. 3.7B) and the shape of colony was round (Fig. 3.7C). Cells of strain JA916^T were rod and oval shaped (0.6 - 1.01 x 1.02 - 1.70 µm; Fig. 3.7D), multiplied by binary fission and were motile by means of a single flagellum (Fig. 3.7E). Transmission electron microphotographs of ultrathin sections of the strain revealed the presence of vesicular type of internal membrane architecture (Fig. 3.7F)

3.2.2.2 16S rRNA gene sequence identities and phylogenetic affiliation (based on 16S rRNA gene and *rpoB* gene sequence)

The BLAST (EzBioCloud) search analysis indicated that strain JA916^T shared highest 16S rRNA gene sequence (1462 bp) identity with *Rba. johrii* JA192^T (98.4%), *Rba. megalophilus* JA194^T (98.3%), *Rba. sphaeroides* ATH 2.4.1^T (98.3%), *Rhodobacter azotoformans* KA25^T (97.9%) and other members of the genus *Rhodobacter* (<97%). The phylogenetic tree (NJ) revealed (Fig. 3.4) that strain JA916^T formed a distinct sub-clade within the clade of *Rhodobacter johrii*, *Rhodobacter megalophilus*, *Rhodobacter sphaeroides* and *Rhodobacter azotoformans.* A similar pattern of clustering was observed when the phylogenetic tree was constructed based on *rpoB* sequences (Fig. 3.8).

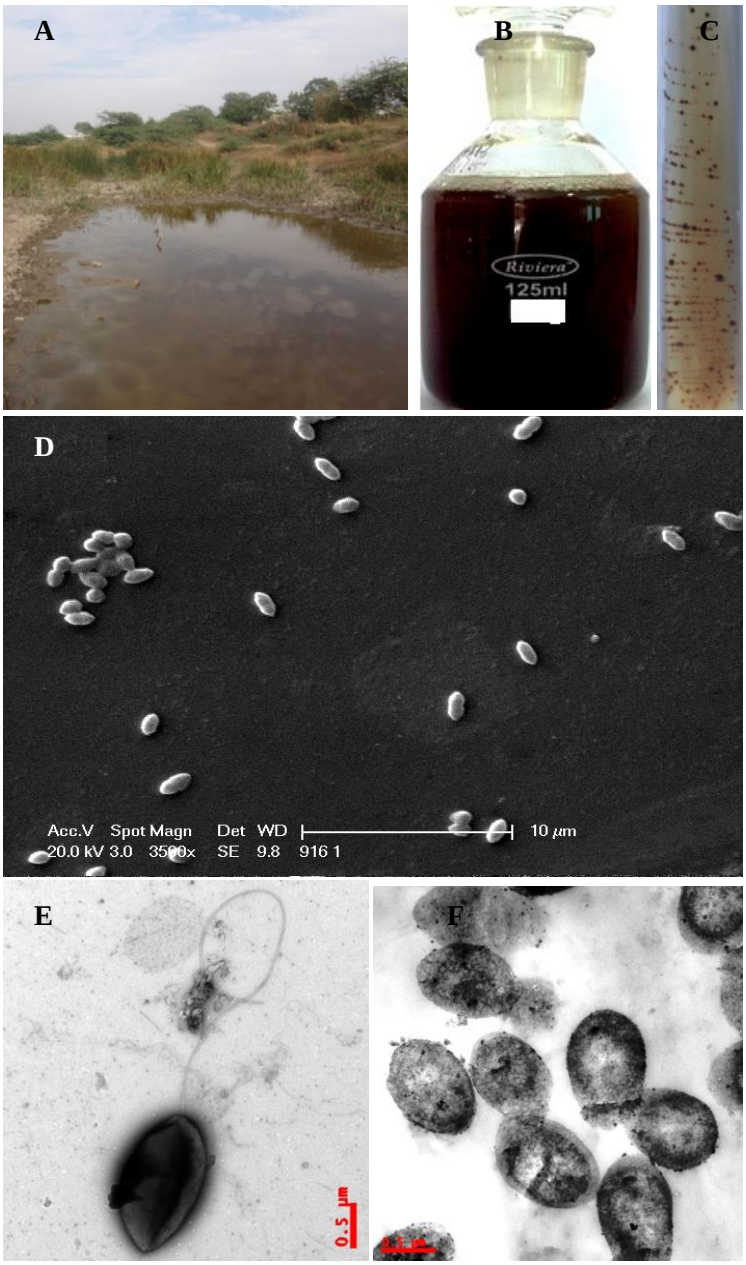


Fig. 3.7 Image showing the characteristics of strain JA916^{T}. 3.6A, sampling site; 3.6B, Image showing the color of phototrophically grown culture; 3.6C, Image showing the color and shape of the colony; 3.6D, Scanning electron micrograph of strain JA916^{T}; 3.6E, Electron micrograph, showing single flagellum of strain JA916^{T}; 3.6F, Electron micrograph of ultrathin sections showing the vesicular architecture of ICM

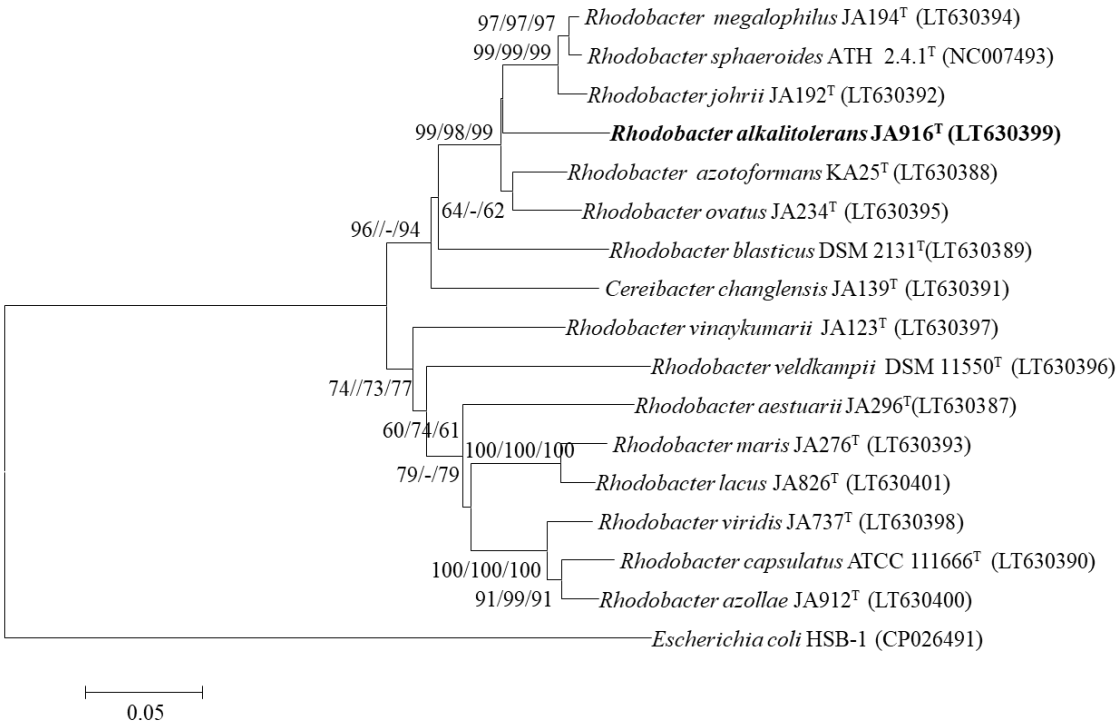


Fig. 3.8 Phylogenetic tree based on *rpoB* gene sequences showing phylogenetic relationship of *Rhodobacter* sp. JA916^T with other members of genus *Rhodobacter*

3.2.2.3 Physiological and biochemical analysis

Strain JA916^T was able to grow photoorganoheterotrophically, photolithoautotrophically and chemoorganoheterotrophically but chemolithoautotrophy and fermentative growth could not be demonstrated. Strain JA916^T did not have any requirement of NaCl for optimum growth but strain can tolerate up to 3%. pH range for growth of strain JA916^T is 6.0-10 with optimum being at 7.0. Strain JA916^T is a mesophile, growing optimally at 30ºC (range 25-35ºC).

Growth was observed with acetate, ascorbate, cysteine, fructose, fumarate, glucose, glycerol, lactate, malate, mannitol, pyruvate, succinate, sucrose, and tartrate; benzoate and glutamate was not utilized by strain JA916^T (Table 3.3).

Table 3.3 Differential characteristics of strain JA916^T and closely related species of the genus *Rhodobacter*

Characteristic	1	2	3	4	5
Cell size	0.6-1.1 x 1.2-1.7	0.8-0.9 x 1.5-1.9	1.2-1.5 x 1.5-2.0	2.0-2.5 x 2.5-3.5	0.6-1.0 x 0.9-1.5
Cell shape	O-R	O-R	O	S-O	O-R
Motility	+	+	-	+	+
Color of cell suspension	YB	YB	YB	GB	YB
Photolithoautotrophy	+	-	+	+	-
Slime production	-	+	+	+/-	+
NaCl requirement	-[b]	-[b]	-	-[b]	-[c]
Growth at pH 10	+	-	-	-	-
pH range	6.0-10.0	6.0-8.0	6.0-8.0	5.0-8.0	6.0-8.0
Growth at 5°C	-	-	-	+	-
Vitamin requirement	b,t[a]	b[a]	t[a]	b,n,t[a]	b,n,t[a]
Carbon sources/e-donors used for growth					
Hydrogen	-	-	-	+	-
Sulfide	+	-	-	+	-
Thiosulfate	-	-	+	-	-
Aspartate	+	+	+	-	(+)
Glutamate	-	+	+	+	+
Tartarate	+	-	+	+	-
Citrate	+	-	-	+	-
G+C content	65.1	65.7	66.7	69.9	69.5
[&]Fatty acid (%)					
$C_{18:1}\omega7c/C_{18:1}\omega6c$	68.4	74	73	75.3	66.8
$C_{18:0}$	9.0	7.9	5.4	7.1	8.9
$C_{15:0}$2OH	4.4	-	-	-	-
$C_{16:0}$	3.8	3.4	4.2	4.6	3.6
$C_{10:0}$3OH	3.1	3.3	3.0	3.8	3.8
$C_{16:1}\omega7c/C_{16:1}\omega6c$	2.3	2.1	1.6	1.9	4.2
$C_{18:1}\omega7c$11 methyl	0.6	1.9	3.4	1.0	3.2
$C_{17:0}$	-	0.6	0.4	0.6	1.2
$C_{19:1}\omega7c/C_{19:1}\omega6c$	1.5	-	-	-	-
$C_{14:0}$3OH/$C_{16:1}$*iso* I	1.8	0.3	0.7	0.4	0.8
iso-$C_{16:0}$	-	-	1.0	-	0.6
$C_{19:0}$cyclo$\omega10c/C_{19:0}\omega6c$	-	-	1.8	1.0	0.8
[&]Isolation	Alkaline brown pond	Rhizosphere soil	Soil	Stagnant water, Sewage plants, Eutrophic ponds, lakes	Photosynthetic sludge for waste water treatment

All the data mentioned above were studied at the authors' laboratory except data taken from Girija et al. (2010)

Species: 1, *Rba. alkalitolerans* JA916^{T}; 2, *Rba. johrii* JA192^{T}; 3, *Rba. megalophilus* JA194^{T}; 4, *Rba. sphaeroides* LMG 2827^{T}; 5, *Rba. azotoformans* JCM 9340^{T}, all strains divide by binary fission, have vesicles as intra cytoplasmic membrane structures, Q10 quinone system and polar lipids phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, glycolipid, aminolipids, diphosphatidylglycerol. Organic substrate utilization was tested during photoheterotrophic growth. All strains utilized malate, acetate, pyruvate, fumarate, and mannitol while none of them utilized benzoate and arginine. All strains were mesophiles, optimally growing at 30-35 $^{\circ}$C and at neutral pH. Symbols: R-O, Rod to oval; R, rod; S-O, spherical to oval; YB, yellowish brown; GB, greenish brown; n, niacin, b, biotin, t, thiamine, +, good growth; -, no growth; (+), weak growth; [a]Data taken from Girija et al. (2010), [b]NaCl not required for growth, but can tolerate up to 3%, [c]NaCl not required for growth, but can tolerate up to 5%

Strain JA916^{T} grew well with ammonium chloride, glutamate and glutamine as nitrogen sources while aspartate, nitrate, nitrite and urea did not support growth. Strain JA916^{T} utilized thiosulfate, sulfate and sulfide as sulfur sources whereas cysteine, elemental sulfur and methionine did not support growth. Strain JA916^{T} showed requirement for biotin and thiamine.

3.2.2.4 Fatty acid composition

$C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{18:0}$, $C_{15:0}2OH$, $C_{16:0}$, $C_{10:0}3OH$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{19:1}\omega7c/C_{19:1}\omega6c$, $C_{14:0}3OH/C_{16:1}$*iso* I and $C_{18:1}\omega7c11$ methyl are the fatty acid profile of the strain JA916^{T}. The differences in the fatty acid composition with the phylogenetic neighbors are given in the Table 3.3.

3.2.2.5 Pigment analysis and quinone composition

The *in vivo* absorption maxima at 377, 476, 509, 595, 800 and 857 nm (Fig. 3.9) indicates the presence of BChl *a* and carotenoids. HPLC analysis indicated the presence of spheroidene as the major carotenoid and ubiquinone-10 (Q10) as predominant quinone system in strain JA916^T as observed with all the other members of the genus *Rhodobacter*.

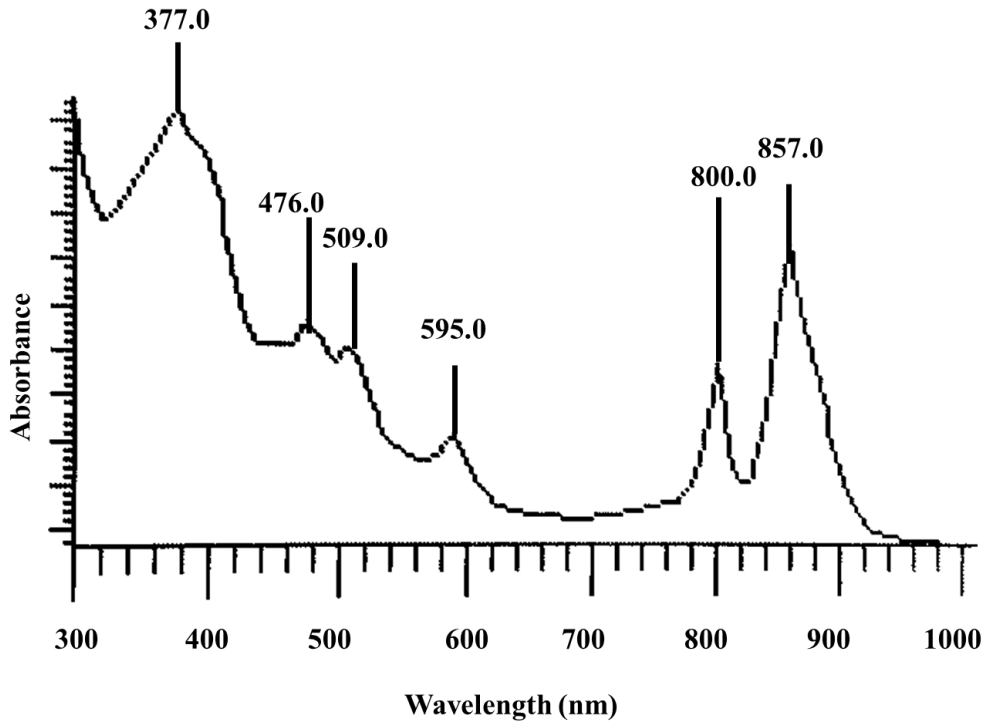


Fig. 3.9 Whole-cell absorption spectrum of strain JA916^T

3.2.2.6 Polar lipid profile

The polar lipid profile of strain JA916^T includes phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), two unidentified phospholipids (PL1,2), unidentified aminolipids (AL1,2), unidentified glycolipids (GL) and four unidentified lipids (UL1,2,3,4) (Fig. 3.10).

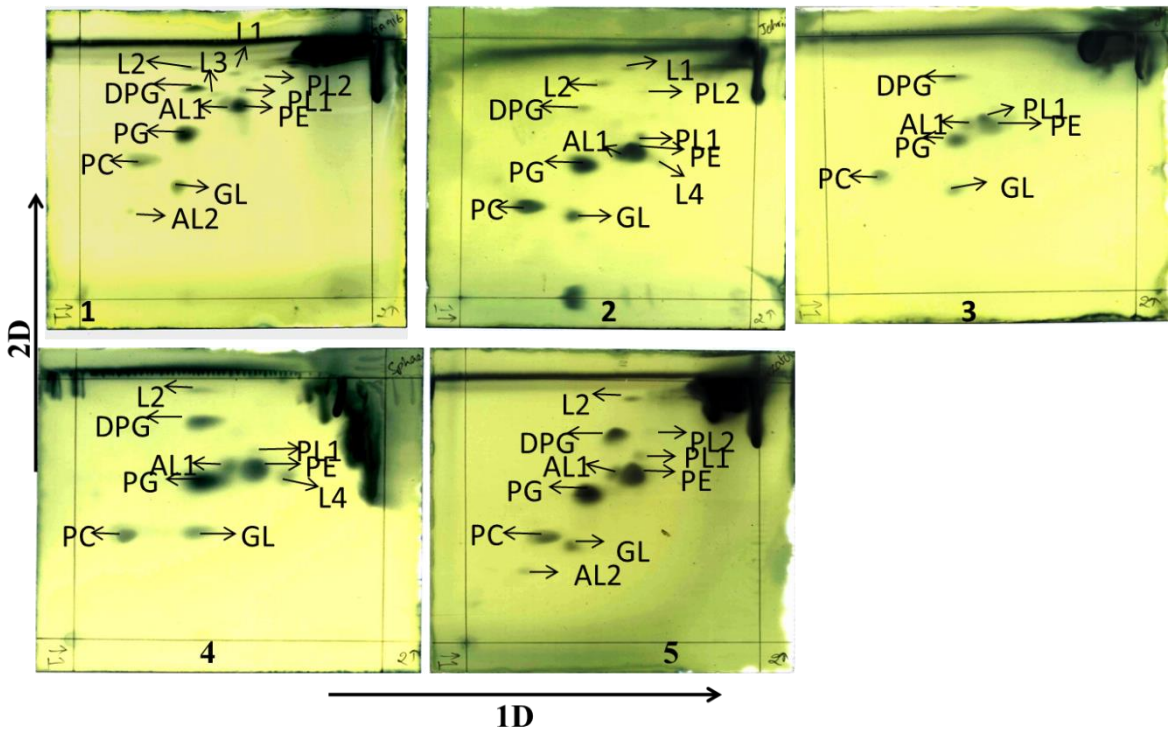


Fig. 3.10 Two-dimensional thin-layer chromatogram showing the polar lipid profile of strain 1. JA916^{T} along with closely related strains 2. *Rba. johrii* JA192^{T}; 3. *Rba. megalophilus* JA194^{T}; 4. *Rba. sphaeroides* DSM158^{T}; 5. *Rba. azotoformans* KA25^{T}. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PL1,2, uncharacterized phospholipids; AL1,2, uncharacterized aminolipids; GL, Glycolipid; APL, aminophospholipid; L1,2,3,4 uncharacterized lipids.

3.2.2.7 DNA-DNA hybridization

For DNA-DNA hybridization studies, when strain JA916^{T} was radioactively labeled, the levels of DNA–DNA re-association with phylogenetically related type strains were less than 40. When the phylogenetically related type strains were labeled and used for DNA–DNA hybridization with JA916^{T} in the reciprocal reaction, the re-association values were less than 38.

3.2.3 Characterization of strain JA983^T

3.2.3.1 Habitat, colony and cell morphology, photosynthetic membrane architecture

Sediment from a fresh water pond (Fig. 3.11A) having a salinity of 0.3% and pH of 6.5-7.0 was collected from Gujarat, India (GPS position: 21°60' N 72°30' E). The colony was round shaped, reddish brown in color but white color could be seen in the periphery when culture grown on plate, cell suspension, color of colony was yellowish brown in color (Fig. 3.11B,C). Cells of strain JA983^T were Gram-stain-negative, oval to rod shaped (Fig. 3.11D), with polar flagella (Fig. 3.11E), divided by binary fission and had vesicular photosynthetic membrane architecture (Fig. 3.11F).

3.2.3.2 Pigment and quinone composition

Intact cells of strain JA983^T showed absorption maxima at 385, 449, 477, 510, 589, 810 and 851nm (Fig. 3.12) indicating the presence of Bchl *a* (absorption maxima at 810 and 851nm) and carotenoids (absorption maxima at 449, 477 and 510). Strain JA983^T has spheroidene as predominant carotenoid. TLC and HPLC analysis indicated the ubiquinone-10 (Q10) as the predominant quinone system.

3.2.3.3 FAME analysis

Strain JA983^T has $C_{18:1}\omega7c/C_{18:1}\omega6c$, $C_{18:0}\omega5c$, $C_{18:0}$ as the major (>10%) fatty acid and $C_{16:0}$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{10:0}3OH$, $C_{15:0}2OH$ and $C_{18:1}\omega7c11$ methyl as minor (>1 but <10%) fatty acids. The fatty acid content and the differences with its phylogenetic neighbors are given in Table 3.4.

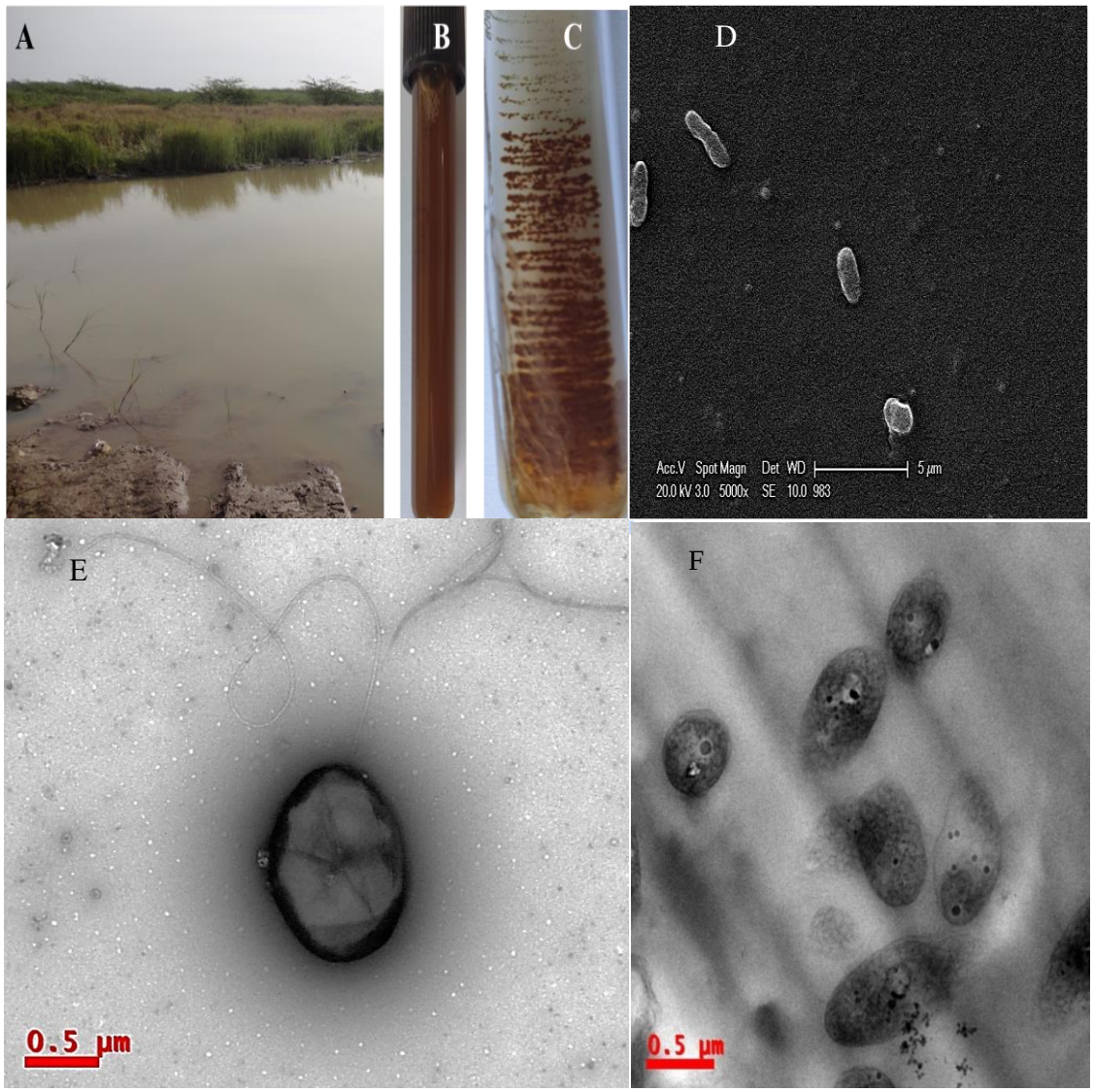


Fig. 3.11 Image showing the characteristics of strain JA983^{T}. A. Sampling site; B, Image showing the color of phototrophic grown culture; C, Image showing the shape of colony Fig. D, scanning electron micrograph of strain JA983^{T}. Bar, 10µm; E. Electron micrograph, showing single flagellum; F. Electron micrograph of ultrathin sections vesicular type of intra cytoplasmic membrane structures

3.2.3.4 16S rRNA gene sequence similarities and phylogenetic affiliation

The results of BLAST analysis indicated that strain JA983^{T} shared highest 16SrRNA gene sequence similarity with *Rhodobacter sphaeroides* ATH2.4.1^{T} (99%),

Rhodobacter johrii JA192^T (99%), *Rhodobacter megalophilus* JA194^T (99%), and other members of the genus *Rhodobacter* (<98.7%).

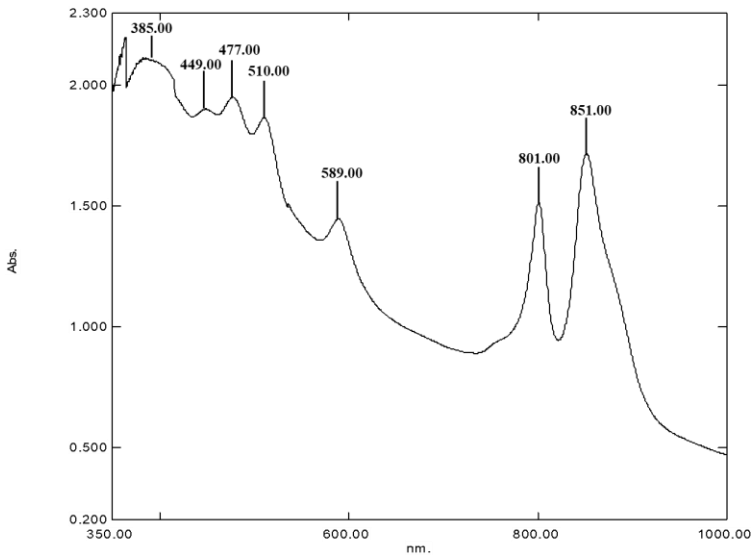


Fig. 3.12 Whole-cell absorption spectrum of strain JA983^T

3.2.3.5 Physiological and biochemical analysis

Strain JA983^T was observed to be utilizing carbon sources like acetate, 2-oxoglutarate, malate, mannitol, glutamate, glycerol, pyruvate, sorbitol, cysteine, formate, propionate, tartrate and ethanol as carbon source or e- donor. Organic carbon sources that could not be utilized were glucose, fructose, sucrose, peptone, casaminoacids, benzoate, methionine, aspartate, ascorbate, lactose, methanol, propanol and L-tryptophan. The variations in the utilization of organic substrates by JA983^T with their phylogenetic neighbours are given in Table 3.4.

Table 3.4 Comparison of characteristics that distinguish strain JA983^T from phylogenetic neighbours

Characteristics	1	2	3	4
Cell size (W x L µm)	0.6-0.9 x 1.0-2.0	2.0-2.5 x 2.5-3.5	1.2-1.5 x 1.5-2.0	0.8-0.9 x 1.5-1.9
Cell shape	O-R	S-O	O	O-R
Motility	+	+	-	+
Color of cell suspension	YB	GB	YB	YB
Photolithoautotrophy	+	+	+	-
Slime production	-	+/-	+	+
NaCl requirement	-¥	-€	-	-€
pH range	6.0- 8.0	5.0-8.0	6.0-8.0	6.0-8.0
Growth at 5 °C	-	-	+	-
Vitamin requirement	t, n	b,n,t	T	B
Carbon sources/e-donors used for growth				
Hydrogen	+	+	-	-
Sulfide	+	+	-	-
Thiosulfate	+	-	+	-
Aspartate	-	-	+	+
2-oxoglutarate	+	-	+	+
Citrate	+	+	-	-
Ethanol	+	+	+	-
Fructose	-	+	+	+
Formate	+	-	+	-
Glucose	-	+	+	+
Propanol	-	+	-	-
Propionate	+	+	+	-
Tartarate	+	+	+	-
Enzyme activities				
Alkaline phosphatase	-	+	-	-
Esterase lipase	+	+	+	-
α-glucosidase	+	-	+	-
Fatty acid profile				
$C_{18:1}\omega7c/C_{18:1}\omega6c$	52.6	75.3	73	74
$C_{18:0}$	13.3	7.1	5.4	7.9
$C_{18:0}\omega5c$	10.7	-	0.5	-
$C_{15:0}2OH$	1.0	-	-	-
$C_{16:0}$	9.5	4.6	4.2	3.4
$C_{10:0}3OH$	2.3	3.8	3.0	3.3
$C_{16:1}\omega7c/C_{16:1}\omega6c$	2.1	1.9	1.6	2.1
$C_{18:1}\omega7c$11 methyl	3.0	1.0	3.4	1.9
$C_{17:0}$	0.6	0.6	0.4	0.6
$C_{14:0}3OH/C_{16:1}$*iso* I	0.5	0.4	0.7	0.3
iso-$C_{16:0}$	-	0.5	1.0	0.4
$C_{19:0}$cyclo$\omega10c/C_{19:0}$	-	1.0	1.8	-

ω6c				
General genomic features				
Genome size (Mbp)	4.22	4.60	4.85	4.59
GC (mol%)	68.8	69.0	68.8	69.1
CDS	4257	4365	4586	4259
RNAs	48	60	52	62

Data present is for the analysis done at author's lab. Taxa;1, *Rhodobacter* sp. JA983^{T}, 2, *Rhodobacter sphaeroides* LMG 2827^{T}; 3, *Rhodobacter megalophilus* JA194^{T}; 4, *Rhodobacter johrii* JA192^{T}. All strains were neutrophiles and mesophiles, have BChl *a* and carotenoids as photosynthetic pigments, Q10 quinone system. All strains have vesicular intracytoplasmic membrane system and polar lipids phosphatidylcholine, phosphatidylglycerol, glycolipid, phosphatidylethanolamine, diphosphatidylglycerol and aminolipids. Under photoheterotrophic growth organic substrate utilization was tested. All strains utilized acetate, fumarate, glutamate, glycerol, malate, mannitol, pyruvate, sorbitol, succinate while none of them utilized arginine and benzoate. All strain showed positive for Oxidase, catalase, esterase (C4), leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase. Negative for valin arylamidase, β-glucoronidase, α-fucosidase, cysteine arylamidase, trypsin, lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase. Symbols: R, rod; R-O, Rod to oval; S-O, spherical to oval; YB, yellowish brown; GB, greenish brown; n, niacin, b, biotin, t, thiamine, +, good growth; -, no growth; ¥NaCl not required for growth, but can grow up to 5%, €NaCl not required for growth, but can grow up to 3%.

Strain JA983^{T} could grow well with glutamine, NH_4Cl, glutamate, aspartic acid and arginine as nitrogen sources but nitrate, nitrite and urea did not support growth. Sulfide, cysteine, methionine, sulfate and thiosulfate were utilized as sulfur

sources by the strain but not sulfite. Thiamine and niacin were required as growth factors for this strain.

Strain JA983^T showed enzyme activities for oxidase, catalase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, α-glucosidase and napthol-AS-BI-phosphohydrolase. But activity of valin arylamidase, β-glucoronidase, α-fucosidase, alkaline phosphatase, cysteine arylamidase, trypsin, lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase could not be observed.

3.2.3.6 Polar lipid profile

Strain JA983^T was found to have glycolipid (GL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), two unidentified aminolipids (AL1, AL2), and two unidentified lipids (L1 and L2) (Fig. 3.13).

3.2.3.7 Genomic information

The draft genome size of strain JA983^T was 4223682 bp, with 4257 protein coding genes and 48 RNAs. The genome was sequenced at approximately above 50X coverage, having 199 total number of contigs in the genome assembly, with N_{50} of 107379 bp. The authenticity of genome was confirmed from the extracted 16S rRNA gene sequence from the assembled genome and compared with Sanger sequencing data. The G+C content calculated from genome was 68.4%.

3.2.3.8 *In-silico* DNA-DNA hybridization and OrthoANI values

The *d*DDH values between strain JA983^T and the type strains of *Rba. sphaeroides*, *Rba. megalophilus* and *Rba. johrii* were 30-45%. The OrthoANI

values between the genome of strain JA983^T and the type strains *Rba. sphaeroides*, *Rba. megalophilus* and *Rba. johrii* were 85-86% (Table 3.5).

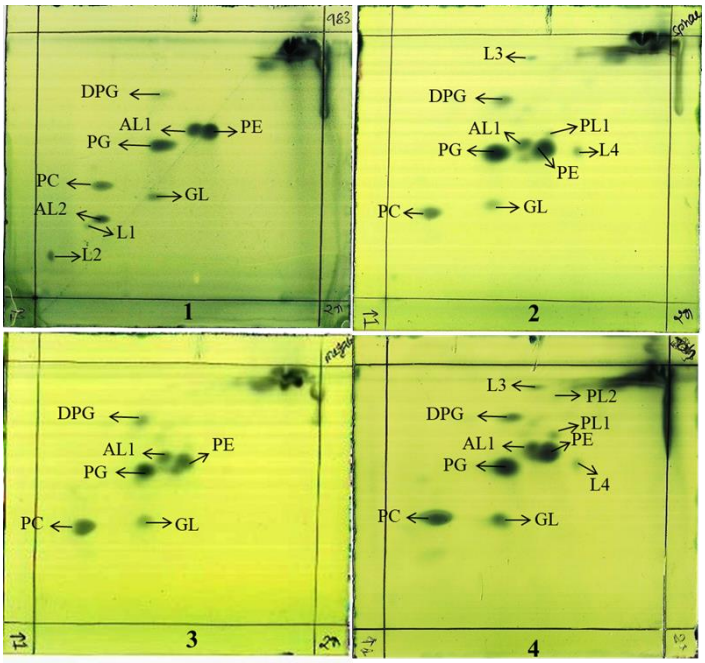


Fig. 3.13 Two-dimensional thin-layer chromatogram showing the polar lipid profile of strain 1. JA983^T along with closely related strains 2. *Rhodobacter sphaeroides* DSM158^T, 3. *Rhodobacter megalophilus* JA194^T, 4. *Rhodobacter johrii* JA192^T; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PL1,2, uncharacterized phospholipids; AL1,2, uncharacterized aminolipids; GL, Glycolipid; APL, aminophospholipid; L1,2 uncharacterized lipids.

Table 3.5 The Ortho ANI (%), *d*DDH (%) values of *Rhodobacter sp*. JA983^T with phylogenetically related strains

Name of the species	OrthoANI (%)	*d*DDH (%)
Rhodobacter megalophilus JA194^T	85.81	30.4
Rhodobacter johrii JA192^T	85.96	44.7
Rhodobacter sphaeroides ATH 2.4.1^T	85.78	30.3

3.3 Reclassification of *Rhodobacter* spp.

3.3.1 Reclassification of *Gemmobacter changlensis* (Basonym: *Rhodobacter changlensis*) into *Cereibacter changlensis*

3.3.1.1 Phylogenetic analysis

In 16S rRNA gene sequence analysis, *Gemmobacter changlensis* JA139^T and some strains (strain JA259, strain JA749, strain WL-71s, strain hswx25 and strain HXG-D9) formed the separate clade with in the genus *Gemmobacter* (Fig. 3.14).

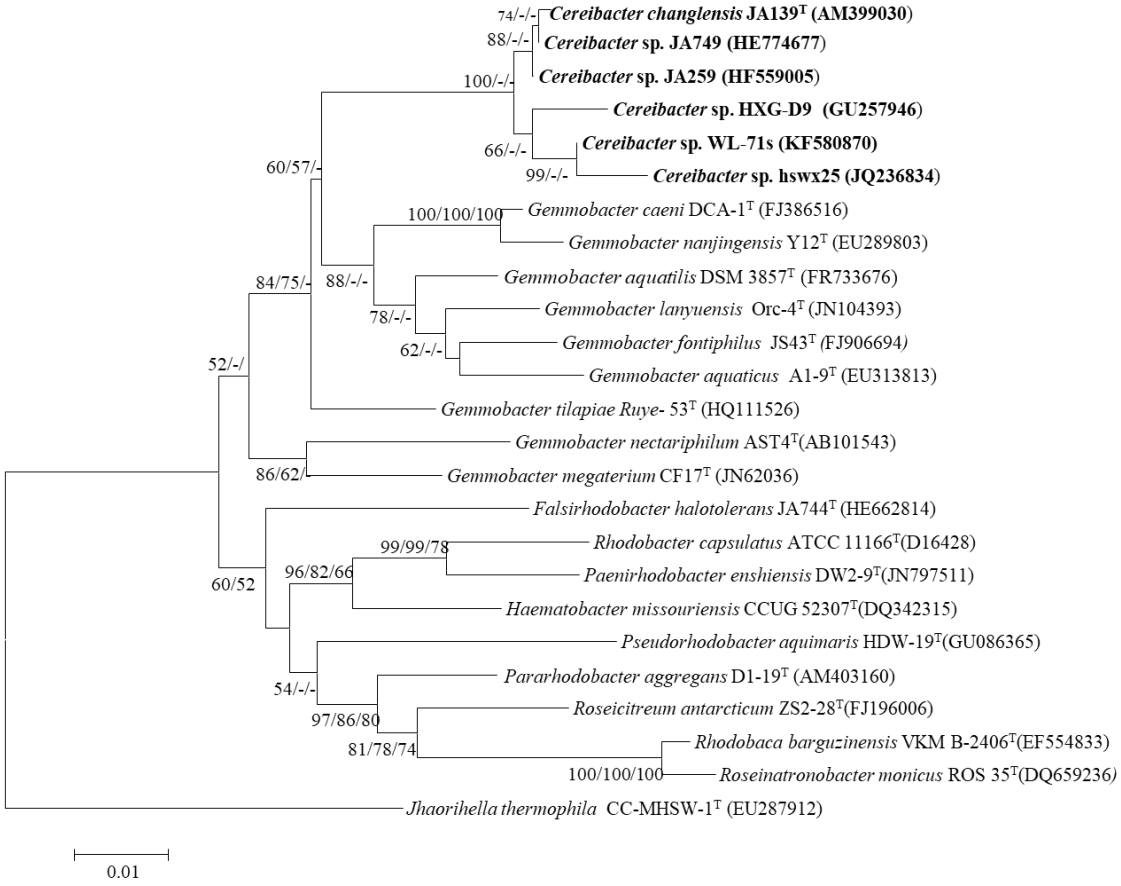


Fig. 3.14 Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between the members of the family 'Rhodobacteraceae'. The tree was computed with MEGA 6.0 software and rooted by using *Jhaorihella thermophila* as the outgroup. position.

3.3.1.2 Polar lipid profile

Phosphatidylglycerol, phosphatidylethanolamine, phosphotidylcholine and an unidentified amino lipid (AL1) are the major polar lipids of all the strains (*Gemmobacter aquatilis*, *Gemmobacter nectariphilus* and *Gemmobacter changlensis*). Apart from the major fatty acids *Gemmobacter aquatilis* and *Gemmobacter changlensis* have diphosphatidylglycerol. And unidentified glycolipid was observed in the *Gemmobacter changlensis* but not in the only G. *nectariphilus* DSM 15620^T and *G. aquatilis* DSM 3857^T (Fig. 3.15).

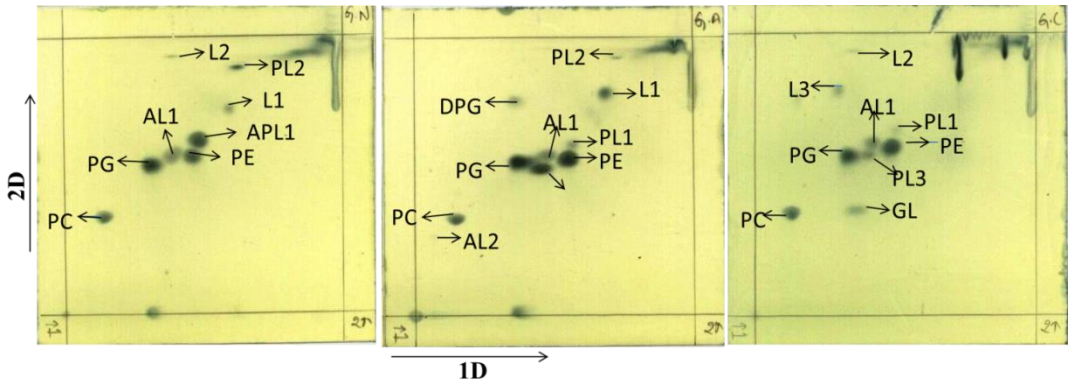


Fig. 3.15 Two-dimensional thin-layer chromatogram of whole cell lipid extracts from *Gemmobacter nectariphilus* DSM 15620^T; 2, *Gemmobacter aquatilis* DSM 3857^T and *Cereibacter chaglensis* JA139^T

3.3.1.3 Physiological analysis

Along with *Gemmobacter changlensis*, genus *Gemmobacter* and some related genera characteristics were compared and given in Table 3.6

Table 3.6 Comparison of *Cereibacter changlensis* JA139^T characteristics with members of closely related genera

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Cell size (µm)	0.8–1.0 x 2.0–4.0	0.3 –1.0 x 1.5–4.0	0.5-2.5 x 0.9-3.5	0.3–0.6 x 0.9–1.2	0.6-1.6 x 1.0 - 4.0	1.0–1.2 x 2–5	ND	0.5-0.8 x 1.5–2.5	0.3-2 x 1-3
Cell shape	O-R	O-R	O-R,C,S	R	R	R	R	R	O-R
Motility	-	+/-	+/-	-	-	+	-	+/-	+/-
Vitamins	b, n, t	b, n, t, paba, B_3, B_{12}	b, n, t, paba, B_{12}	b, n, t, paba, B_3	ND	-	ND	B_3,B_{12}	b, n, t, paba, B_{12}
Phototrophic growth	+	-	+	-	-	-	-	-	+
BChl *a*	+	-	+	-	-	-	-	-	+
Internal membrane system	Vesicles	-	vesicles/ lamellae	-	-	-	-	-	Vesicles
Production of carotenoids	+	-	+	-	-	+	-	-	+
Light harvesting complexes	LHI, LH II	-	LHI, LH II	-	-	-	-	-	LHI, LH II
Growth at 5 c	+	+/-	-	+	-	-	-	-	-
NaCl requirement	-	+/-	-	-	+	-	+	-	+
Nitrate reduction	-	+/-	¥+/-	-	-	ND	-	-	-
Dark anaerobic growth	+	+/-	+	+	-	-	-	+	+
Dark aerobic growth	+	+	+	-	-	+	+	+	+

Polar lipids									
DPG	+	+/-	+	-	-	+	-	ND	$^{\epsilon}$-
GL	+	+/-	+/-	-	-	+	-	ND	$^{\epsilon}$-
PC	+	+	+	-	+	+	-	ND	$^{\epsilon}$-
Fatty acids									
$C_{18:0}$3OH	-	+	+/-	+	-	-	-	+/-	-
$C_{18:1}\omega 9c$	-	+/-	-	-	-	-	+	ND	-
G+C content	69.2 – 69.4	61.2 – 69.3	62.8 -73.6	67.2	56.6-58	74-76	65-65.5	65.1-68	58-69
Isolation source	Snow sample	fresh water	fresh water and marine	Soil	Marine	Saltern	Blood	marine aquarian system	hypersalie and marine

Taxa: 1,*Cereibacter changlensis* JA139^{T} (data from this study); 2, *Gemmobacter* (data for 9 species from Chen et al., 2013b; Liu et al., 2014); 3, *Rhodobacter* (unless indicated, data for 12 species from Girija et al., 2010; Imhoff et al., 1984; Raj et al., 2013; Srinivas et al., 2007; Subhash et al., 2013); 4, *Paenirhodobacter* (*Paenirhodobacter enshiensis* DW2-9^{T} Wang et al., 2014)*; 5, Pseudorhodobacter* (*Pseudorhodobacter aquimaris* HDW-19^{T}, Uchino et al., 2002; *P. ferrugineus* ATCC 25652^{T}, Jung et al., 2012); 6, *Falsirhodobacter* (*Falsirhodobacter halotolerans* JA744^{T}, Subhash et al., 2013); 7, *Haematobacter* (*Haematobacter massiliensis Framboise*^{T} and *H. missouriensis* H189^{T}; Helsel et al., 2007); 8, *Pararhodobacter* (*Pararhodobacter aggregans* D1-19^{T} , Foesel et al., 2011); 9, *Rhodovulum* (data from 16 species; Lakshmi et al., 2011; Srinivas et al., 2012, 2014). OR, oval to rod; R, rod; b, Biotin; B_{12}, vitamin B_{12}; B_{12}, vitamin B_3; n, niacin; paba, p-aminobenzoic acid; t, thiamine; LH, light harvesting system;$^{¥}$variable at strain level; $^{\epsilon}$Data not available for *Rhodovulum iodosum, R. euryhalinum, R. adriaticum, R. lacipunicei, R. marinum, R. robiginosum, R.imhoffii;* +, present; -, absent; ND, No data available

3.3.2 Reclassification of *Rhodobacter* members into three new genera

3.3.2.1 Phylogenetic analysis using 16S rRNA gene sequences

Based on 16S rRNA gene sequence phylogenetic analysis, the sixteen species of the genus *Rhodobacter* form five distinct clades (Fig. 3.16) with interspersing chemotrophs (members of other described genera in the family 'Rhodobacteraceae'). Clade I, the *Rba. sphaeroides* clade, contains *Rba. sphaeroides*, *Rba. johrii*, *Rba. megalophilus*, *Rba. azotoformans*, *Rba. ovatus* and *Rba. alkalitolerans.* Clade II contains the *Rba. capsulatus* clade, which includes *Rba. capsulatus, Rba. viridis, Rba. azollae, Rba. sediminis*, *Rba. aestuarii*, *Rba. maris* and *Rba. lacus.* Clade III, the *Rba. blasticus*, clade contains only a single species, *Rba. blasticus.* Clade IV is the *Rba. veldkampii* clade containing only *Rba. veldkampii* while clade V is represented by *Rba. vinaykumarii.* The 16S rRNA gene pairwise sequence similarities among *Rhodobacter* species were calculated using LALIGN tool (https://www.ebi.ac.uk/Tools/psa/lalign/) and the results showed that some of the *Rhodobacter* species (between clade I and clade II) have 94.0% sequence similarity (Table 3.7), which is less than the recommended value for genus delineation (Rosselló-Móra and Amann, 2015). Phylogenetic analysis based on 92 bacterial core genes (UBCG) also showed (Fig. 3.17) that members of *Rhodobacter* are not monophyletic, as observed in the 16S rRNA gene-based phylogenetic tree (Fig. 3.16), with the exception of *Rba. veldkampii* and *Rba. vinaykumarii* forming one cluster unlike in the 16S rRNA gene-based phylogenetic tree.

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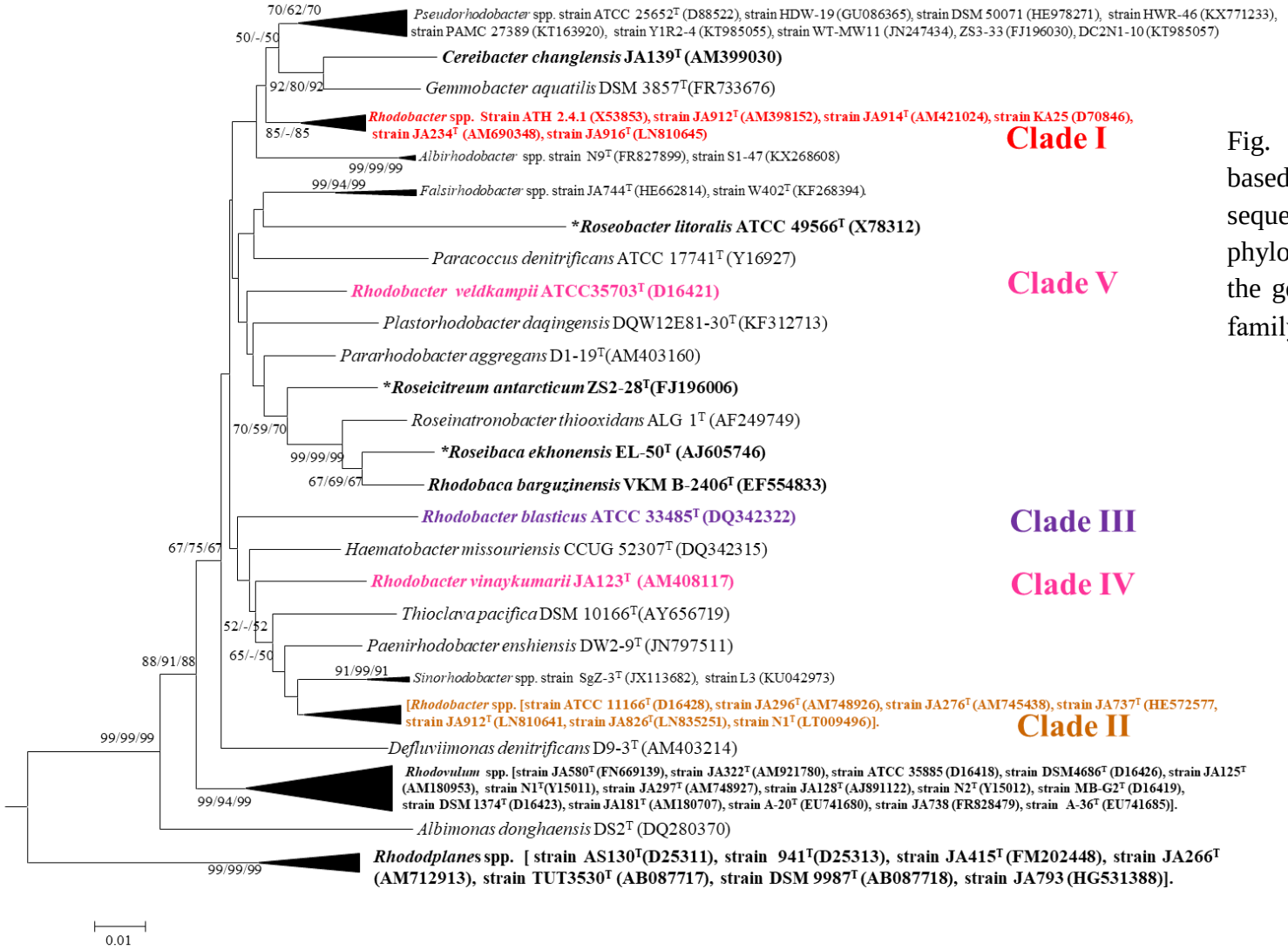

Fig. 3.16 Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of the genus *Rhodobacter* of the family 'Rhodobacteraceae'.

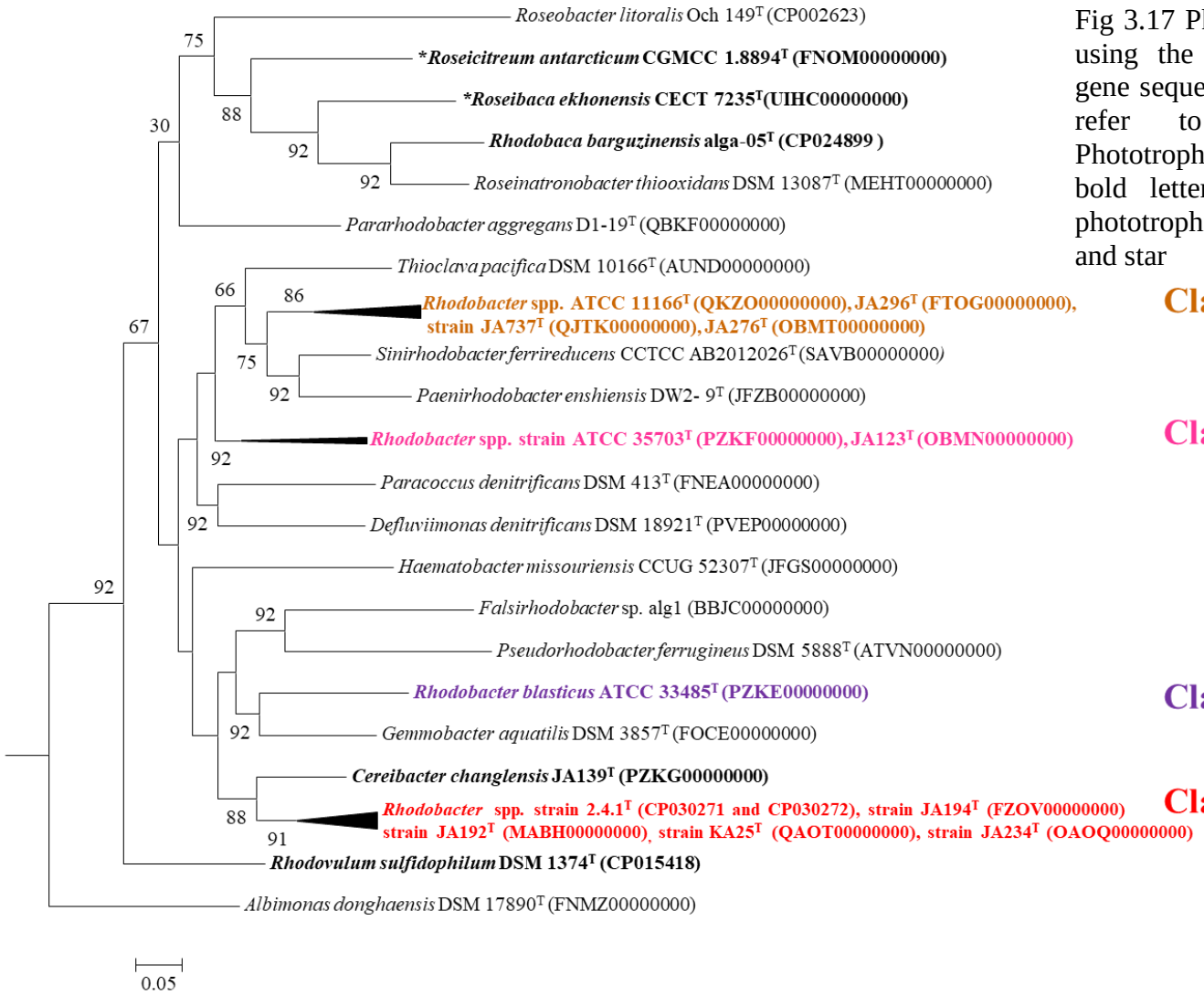

Fig 3.17 Phylogenetic tree constructed using the Ninety-two bacterial core gene sequences. Bootstrap percentages refer to NJ/ML/ME analysis. Phototrophic bacteria are indicated by bold letters and aerobic anoxygenic phototrophic bacteria with bold letters and star

Table 3.7 16S rRNA gene sequence pairwise similarity percentages among the genus *Rhodobacter*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	100															
2	100	100														
3	99.9	99.9	100													
4	98.1	98.1	98.1	100												
5	98.5	98.5	98.5	98.8	100											
6	98.4	98.4	98.5	97.5	98.1	100										
7	95.6	95.6	95.7	95.4	95.5	95.8	100									
8	95.7	95.7	95.7	94.8	95.0	94.7	95.7	100								
9	95.8	95.8	95.8	94.8	95.1	94.8	95.6	99.5	100							
10	95.4	95.4	95.4	94.4	94.7	94.4	95.3	99.5	99.9	100						
11	94.9	94.9	94.9	93.8	94.1	93.8	94.6	98.9	99.3	99.4	100					
12	95.1	95.1	95.1	94.4	94.8	95.0	94.4	96.8	97.0	96.7	96.2	100				
13	95.0	95.0	94.9	94.0	94.8	94.5	94.9	97.5	97.3	97.1	96.4	97.8	100			
14	93.9	93.9	93.9	92.8	93.8	93.5	93.7	96.5	97.0	97.0	96.9	96.8	98.4	100		
15	94.8	94.8	94.7	94.8	94.7	94.7	94.6	94.8	94.7	94.5	94.0	95.6	95.0	94.5	100	
16	95.9	95.9	95.9	95.0	95.6	95.5	95.2	96.3	96.4	96.1	95.5	97.8	96.5	95.9	95.9	100

1. *Rhodobacter sphaeroides* 2.4.1^T; 2- *Rhodobacter megalophilus* JA194^T; 3- *Rhodobacter johrii* JA192^T; 4- *Rhodobacter azotoformans* KA25^T, 5- *Rhodobacter ovatus* JA234^T; 6- *Rhodobacter alkalitolerans* JA916^T; 7- *Rhodobacter blasticus* DSM 2131^T: 8-*Rhodobacter capsulatus* ATCC 11166^T; 9- *Rhodobacter viridis* JA737^T; 10- *Rhodobacter sediminis* N1^T; 11- *Rhodobacter azollae* JA912^T; 12- *Rhodobacter aestuarii* JA296^T; 13- *Rhodobacter maris* JA276^T; 14- *Rhodobacter lacus* JA826^T; 15- *Rhodobacter veldkampii* ATCC 35703^T; 16- *Rhodobacter vinaykumarii* JA123^T.

Clade I members typically have large genomes (4.3-4.7 Mb) and high G+C content 68.2-69.1 mol%). An exception in Clade I is *Rba. ovatus,* which has an 3.8 Mb genome and genomic GC content of 66.5 mol%. Clade II members have 3.6-3.9 Mb genomes and genomic GC content of 61.0-66.6 mol%. Clade III members have a genome size of 3.6-3.7 Mb, 66.4-66.5 GC mol%, while clade IV and V have 3.3, 3.5 Mb and 65.0, 68.2 G+C mol%, respectively (Fig. 3.18 and Table 3.8)

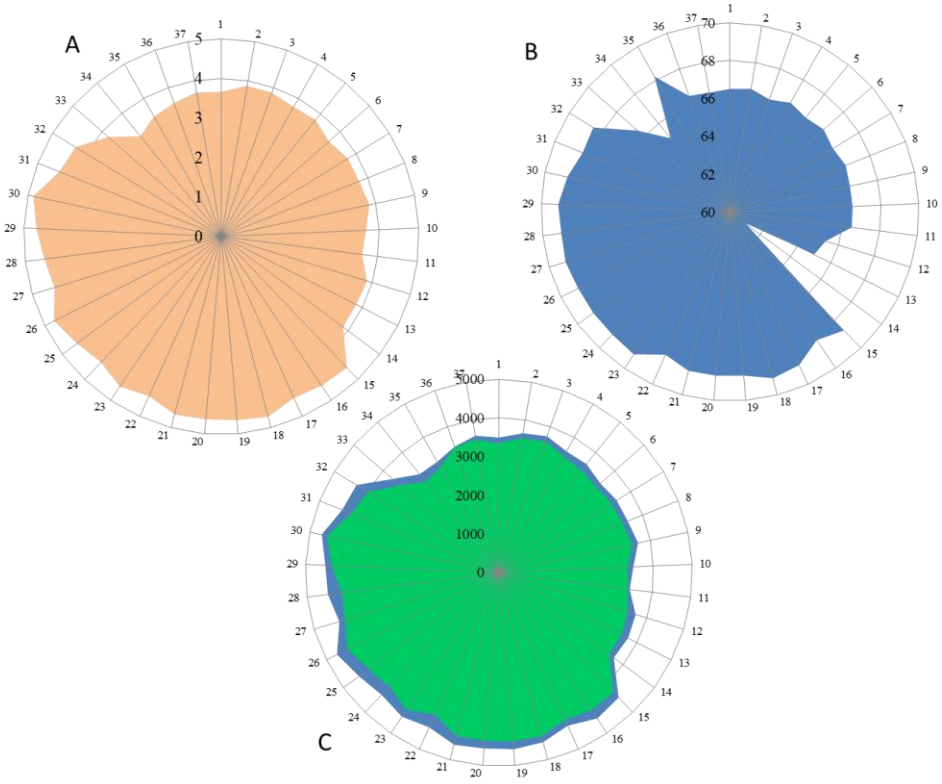

Fig. 3.18 Genomic information of genus *Rhodobacter* members. A, Genome size (Mb); B, G+C content calculated from Genome in mol %; C, No of genes in blue colour and no of proteins in green colour. Each spoke in the circle represent one strain and the numbering same as in Table 3.8.

Table 3.8 Genomic information of species/strains of genus *Rhodobacter*

S. No	Organism name	Size (Mb)	GC%	Genes	No. of proteins
	***Rhodobacter sensu stricto* clade**				
1	*Rba. capsulatus* DSM 1710^T	3.66	66.5	3495	3346
2	Strain SB1003	3.87	66.6	3660	3531
3	Strain DSM 938	3.86	66.3	3742	3593
4	Strain DE442	3.76	66.6	3593	3446
5	Strain B6	3.80	66.4	3607	3403
6	Strain YW1	3.64	66.6	3497	3354
7	Strain YW2	3.77	66.4	3576	3414
8	Strain R121	3.76	66.6	3588	3446
9	Strain Y262	3.84	66.5	3683	3528
10	Strain B41	3.67	66.5	3480	3331
11	Strain A52	3.60	66.5	3411	3357
12	*Rba. viridis* JA737^T	3.86	65.3	3701	3481
13	*Rba. maris* JA276^T	3.83	65	3745	3557
14	*Rba. aestuarii* JA296^T	3.84	61	3695	3544
	***Rba. sphaeroides* Clade**				
16	*Rba. sphaeroides* ATH 2.4.1^T	4.60	68.7	4474	4280
17	Strain ATCC 17025	4.55	68.2	4547	4280
18	Strain ATCC 17029	4.48	68.9	4338	4170
19	Strain KD131	4.71	69.1	4530	4367
20	Strain MBTLJ-8	4.66	68.7	4580	4374
21	Strain MBTLJ-13	4.64	68.7	4561	4360
22	Strain MBTLJ-20	4.65	68.7	4596	4365
23	Strain EBL0706	4.43	68.3	4387	4024
24	Strain AB25	4.58	69.1	4489	4279
25	Strain AB27	4.4	69.0	4381	4084
26	Strain AB29	4.54	69.0	4502	4192
27	Strain AB24	4.74	69.0	4692	4389
28	Strain WS8N	4.41	69.1	4317	4164
29	*Rba. johrii* JA192^T	4.51	69.0	4458	4093
30	Strain CDR-SL7Cii	4.68	69.1	4496	4330
31	*Rba. megalophilus* JA194^T	4.86	68.8	4690	4552
32	*Rba. azotoformans* KA25^T	4.41	68.4	4369	4112
33	Strain YLK20	4.32	68.5	4327	3980
34	*Rba. ovatus* JA234^T	3.81	66.5	3668	3422
	***Rba. veldkampii* Clade**				
35	*Rba. veldkampii* DSM 11550^T	3.26	65	3273	3019
36	*Rba. vinaykumarii* JA123^T	3.48	68.2	3281	3122
	***Rba. blasticus* Clade**				
37	*Rba. blasticus* DSM 2131^T	3.58	66.5	3455	3441
	Strain 28/5	3.70	66.4	3595	3494

3.3.2.2 Genomic features of *Rhodobacter* spp.

Out of sixteen type strains of *Rhodobacter*, genome sequences of twelve type strains are available. Genome sequences of *Rba. alkalitolerans* (Gandham et al., 2018), *Rba. azollae, Rba. lacus* (Suresh et al., 2017) and *Rba. sediminis* (Subhash and Lee, 2016) are not available. A genomic summary of the twelve species (and some related strains) is presented in Fig. 3.18

3.3.2.3 Analysis of core and pan-genome of the genus *Rhodobacter*

Bacterial pan-genome analysis was carried out between the type strains of the genus *Rhodobacter*. In the 12 analyzed species of the genus *Rhodobacter*, 1239 genes were identified as core genes (Fig. 3.19). The species of the *Rba. sphaeroides* clade have 2294 genes as a core genome with 6256 genes as accessory (Fig. 3.20), whereas species of the *Rba. capsulatus* clade share 2225 genes as a core genome with 2588 genes as the accessory genome (Fig. 3.21). The distribution of core genome, accessory genome and unique genes is given in Table 3.9

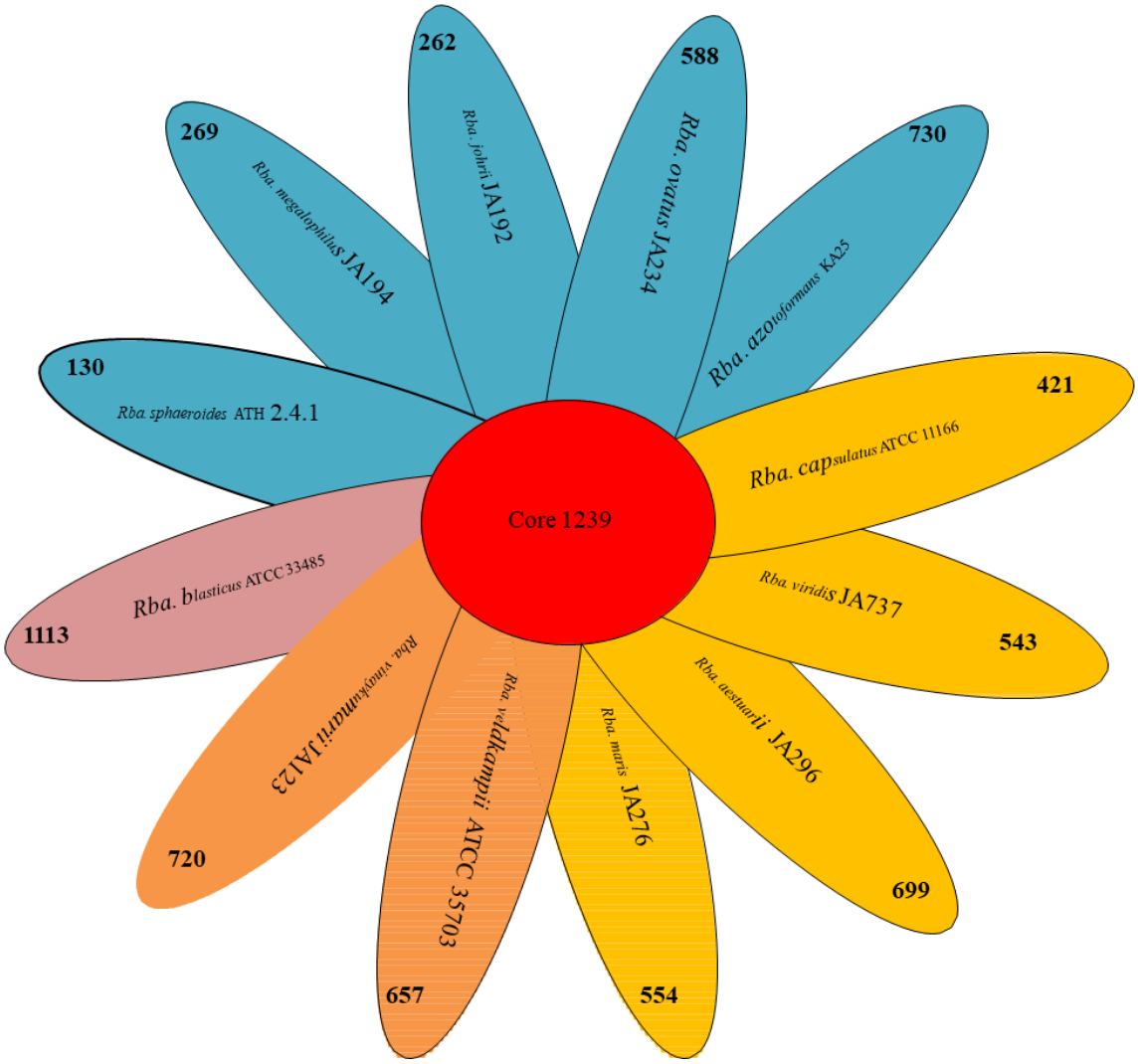

Fig. 3.19 Pan-genome analysis of *Rhodobacter* members. Floral plot showing the number of core genes in the center of flower and number in each petal represent unique genes in each species

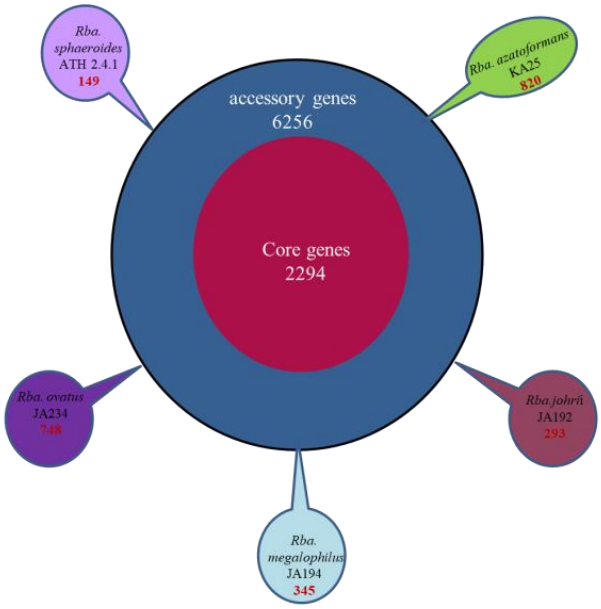

Fig. 3.20 Pan-genomic analysis of *Rba. sphaeroides* clade, Inner pink circle representing the core genes. Outer blue circle representing the accessory genes of all 5 organisms, and the buds representing the individual organisms' unique genes

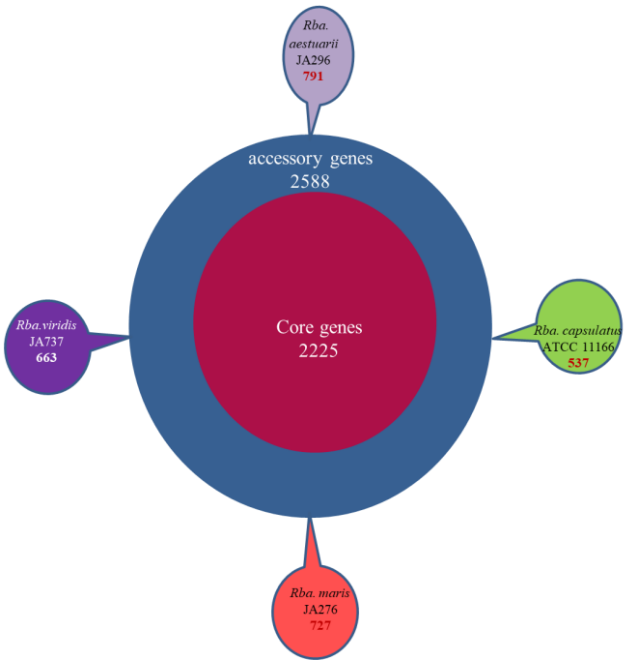

Fig. 3.21 Pan-genomic analysis of *Rba. capsulatus* clade, inner pink circle representing the core genes. Outer blue circle representing the accessory genes of all 4 organisms, and the buds representing the individual organisms' unique genes

Table 3.9 BPGA of all 12 species of genus *Rhodobacter*

Organism name		core genes	accessory genes	unique genes	absent genes
12 species of genus *Rhodobacter*					
	Rba. aestuarii JA296	1239	1655	699	9
	Rba. azotoformans KA25	1239	2233	730	7
	Rba. blasticus ATCC 33485	1239	1127	1113	57
	Rba. capsulatus ATCC11166	1239	1791	421	2
	Rba. johrii JA192	1239	2594	262	34
	Rba. maris JA276	1239	1832	554	5
	Rba. megalophilus JA194	1239	2961	269	0
	Rba. ovatus JA234	1239	1655	588	8
	Rba. sphaeroides ATH 2.4.1	1239	2478	130	3
	Rba. veldkampii ATCC 35703	1239	1127	657	48
	Rba. vinaykumarii JA123	1239	1184	720	26
	Rba. viridis JA737	1239	1776	543	8
Pan-genome analysis of *Rba. sphaeroides* clade	*Rba. azotoformans* KA25	2294	1082	820	66
	Rba. johrii JA192	2294	1508	293	90
	Rba. megalophilus JA194	2294	1827	345	2
	Rba. ovatus JA234	2294	437	748	531
	Rba. sphaeroides ATH 2.4.1	2294	1402	149	30
Pan-genome analysis of *Rba. capsulatus* clade	*Rba. aestuarii* JA296	2225	571	791	130
	Rba. capsulatus ATCC11166	2225	684	537	53
	Rba. maris JA276	2225	665	727	87
	Rba. viridis JA737	2225	668	663	80
Pan-genome analysis of *Rba. veldkampii* clade	*Rba. vinaykumarii* JA123	2014	0	1123	1004
	Rba. veldkampii ATCC 35703	2014	0	1004	1123

3.3.2.4 Digital DNA-DNA hybridization and Average Nucleotide Identity (ANI)

*d*DDH values were calculated between 12 species of genus *Rhodobacter* and other closely related genera. *d*DDH values ranged from 18.6 to 59.0% between type strains of the genus *Rhodobacter* (Table 3.10) except between the pair of *Rba. sphaeroides* 2.4.1^T and *Rba. megalophilus* JA194^T (81.6%). The results of *d*DDH also matched with the data obtained using the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker, 2019). Orthologous Average Nucleotide Identity (OrthoANI) varied between 68.25-94.93% between type strains of the genus *Rhodobacter*. The only exception was again the pair, *Rba. sphaeroides* 2.4.1^T and *Rba. megalophilus* JA194^T, where it was 97.96% (Table 3.10).

3.3.2.5 POCP and AAI values for genera delineation

A POCPs threshold below 50% was proposed to determine if a species belongs to the two different genera (Qin et al., 2014). The POCP values within the genus *Rhodobacter* ranged from 54.2% to 88.2%. The lowest POCP value was between *Rba. megalophilus* JA194^T and *Rba. aestuarii* JA296^T and highest POCP value was between *Rba. megalophilus* JA194^T and *Rba. sphaeroides* 2.4.1^T. The POCP values among the clade I species ranged from 66.5% to 88.2%, whereas for clade II values were between 72.1% to 79.9%, and was 71.1% between *Rba. veldkampii* and *Rba. vinaykumarii*. The POCP value between *Rba. capsulatus* ATCC 11166^T and *Rba. sphaeroides* 2.4.1^T was 59%. POCP values among *Rhodobacter* clades and related genera are above 50% (Table 3.11).

The AAI among the different clades of *Rhodobacter* along with chemotrophs were below 80% (Table 3.11). The AAI values within the genus *Rhodobacter* ranged from 63.04 to 98.56%. The lowest AAI value was between *Rba. megalophilus* JA194^T

and *Rba. aestuarii* JA296^T and highest AAI value was between *Rba. megalophilus* JA194^T and *Rba. sphaeroides* 2.4.1^T. The AAI values in clade I range from 98.56% to 80.37%, whereas clade II values range from 77.86% to 89.21%, and 72.85% between *Rba. veldkampii* and *Rba. vinaykumarii*. The AAI value between the *Rba. capsulatus* ATCC 11166^T and *Rba. sphaeroides* 2.4.1^T was 64.36%. AAI values between the type species of all the genera under study were below the recommended cut-off value of 80% (Luo et al., 2014; Table 3.11). The average AAI value between the members of clade I and clade II was 64.1%, between clade I and clade III was 66.64%, between clade I and clade IV was 67.18%, between clade II and clade III was 63.72%, between clade II and IV was 68.53% and between the clade III and IV was 65.14%.These values are well below the recommended cut-off of 80% used for the genus delineation (Luo et al., 2014).

3.3.2.6 Analysis of photosynthesis related genes of *Rhodobacter* spp.

3.3.2.6.1 PufLM protein phylogenetic analysis

PufLM proteins are essential components of bacteria which perform photosynthesis with the help of photosystem II type of photosynthetic apparatus, and the phylogeny of both anaerobic and aerobic photosynthetic bacteria can be studied by PufLM protein analysis (Imhoff et al., 2018). Full length amino acid sequences of PufLM of 12 *Rhodobacter* type strains were extracted from their genomes and a phylogenetic tree was (Fig. 3.22) constructed using *R. sulfidophilum* as the out-group. All the *Rhodobacter* members were recovered in 4 different clades, similar to the 16S rRNA gene based phylogenetic tree although *Rba. veldkampii* and *Rba. vinaykumarii* formed a single cluster, as in the phylogenomic tree.

Table 3.10 ANI and *d*DDH values of genus *Rhodobacter* members and some related genera

***d*DDH values**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1		88.04	79.5	77.84	73.92	74.13	73.81	74.01	74.13	75.17	75.62	73.7	74.11	74.02	70.97	71.19	76.13	70.75	75.18	74.08	70.31	70.69	72.28	71.26	73.58	71.43	73.78	69.89	64.07	66.21	78.13	71.14
2	33.9		79.38	77.53	73.95	74.09	74.13	74.11	74.03	75.13	75.3	73.56	74.09	73.78	71.05	71.94	75.9	70.78	75.01	73.86	73.49	70.08	71.97	70.9	73.34	71.39	73.38	69.58	64.2	66.29	78.04	70.81
3	22.1	21.9		79.81	73.54	73.9	73.63	73.64	73.49	75.4	74.77	72.68	73.66	73.49	70.59	71.81	75.21	70.67	75.01	73.39	72.81	70.01	71.54	70.48	72.71	71.09	73.1	69.3	64.16	65.59	77.72	70.71
4	20.3	20.2	22		71.84	72.76	72.04	72.11	72.38	68.25	73.62	71.58	71.93	72.21	70.18	70.44	73.73	70.65	73.63	72.56	71.75	69.9	71.15	70.43	71.29	70.2	74.2	69.08	64	65.27	75.43	70.3
5	19.3	19.5	19.1	19.2		94.9	97.96	85.88	81.21	74.95	74.59	74.67	79.25	74.5	72.5	73.13	74	71.63	73.8	75.19	73.91	70.52	72.53	71.28	74.48	72.11	74.05	69.58	63.65	66.04	75.24	71.21
6	19.2	19.2	18.8	20.1	58.9		94.93	86.03	81.28	75.42	74.68	74.4	79.17	74.34	72.74	73.04	74.36	71.44	73.62	75.3	74.16	70.84	72.69	71.08	74.75	72.12	74.24	69.67	63.46	66.27	75.36	71.49
7	19.1	19.3	19.2	19.1	81.6	59		85.75	81.01	75.22	74.89	74.47	78.98	74.52	72.56	73.28	73.99	71.61	74.06	75.65	74.25	70.67	72.72	71.11	74.45	72.3	74.2	69.6	63.41	66.47	74.95	71.24
8	19.6	19.6	19.2	18.7	30.3	30.6	30.4		81.77	75.43	74.62	74.69	78.9	74.23	72.59	73.23	74.16	71.58	73.77	75.28	73.92	70.56	72.78	71.32	74.49	72.45	74.18	69.67	63.97	66.9	75.28	71.85
9	19.1	19.1	18.9	20.1	24.3	24.4	24.2	24.8		75.71	75.41	74.9	78.74	74.44	72.97	73.33	73.63	72.19	73.22	75.99	73.56	71.38	73.31	71.53	73.97	71.85	74.16	69.96	63.76	66.52	74.37	72.11
10	20	19.7	21.2	20.9	20.3	20.6	20.7	20.5	21		76.95	73.9	74.93	74.7	71.49	72.99	74.94	70.65	75.11	74.38	74.28	71.21	73.03	71.52	74.66	72.29	75.1	70.62	64.43	66.66	76.01	72.06
11	19.3	19.3	19.4	18.8	19.2	19.7	20	19.5	19.7	21		74.25	75.09	74.64	71.74	72.56	74.25	71.89	74.02	74.86	73.76	71.48	73.23	72	73.65	71.62	74.47	69.8	64.56	65.97	75.56	71.7
12	19.3	18.9	18.7	18.6	19.4	19.3	19.3	19.3	19.3	19.4	19.1		74.98	73.69	71.74	72.02	72.74	71.88	72.28	75.71	73.55	70.71	72.59	71.57	73.04	71.23	73.64	69.62	64.27	66.45	73.58	71.33
13	19.2	19.1	18.6	18.6	22.1	22.1	22.1	22.1	21.7	20.2	19.4	19.4		74.89	72.91	73.26	74.3	72.54	73.9	76.67	74.48	71.24	73.2	71.85	74.57	72.42	74.34	70.25	64.2	66.71	75.04	71.6
14	18.8	19.3	19.8	18.9	19.3	19.4	19.6	19	19.1	20.1	19.5	18.9	19.2		71.52	72.6	73.67	71.27	73.87	74.18	73.83	70.65	72.75	71.26	73.83	72.08	74.12	70.11	64.11	66.23	74.53	71.57
15	19.7	19.9	19.2	19.3	19.6	19.2	19.3	18.7	19	19.5	18.4	18.9	18.9	19.2		71.07	71.01	70.55	70.82	72.62	71.18	69.65	71.05	70.23	70.69	69.54	71.22	68.81	63.89	65.11	71.57	69.91
16	19.1	19.3	19.4	19.6	19.9	19.5	20.3	19.5	20.7	20.2	19.2	18.8	19.3	13.9	19.3		72.46	69.9	72.22	72.6	72.31	70.08	71.61	70.17	72.13	71.03	73.04	69.16	64.1	66.05	72.59	70.31
17	19.9	20	20	18.7	19.6	19.5	19.5	19.4	19.3	20	19	18.8	19.3	19.1	18.4	19.5		70.07	75.01	73.25	73.38	70.08	71.63	70.56	73.46	71.72	74.43	69.31	64.43	66.74	79.05	70.76
18	18.4	18.4	18.4	17.8	19.6	19.1	18.8	18.5	18.4	18.4	18.1	18.1	17.8	17.7	18.7	18.9	17.7		70.51	72.85	70.57	70	71.38	70.5	70.32	69.07	70.39	69.31	63.29	63.9	70.71	70.42
19	19.4	19.2	19.5	18.9	19.6	19.2	19.6	18.8	18.6	21.4	19.2	18.8	19.1	19.3	18.8	19.3	19.5	18.2		73.36	73.35	70.31	72.11	70.9	73.27	71.77	73.55	70.14	63.93	66.14	76.67	71.13
20	18.9	19	19.1	18.3	19.5	19.5	19.9	19.6	19.8	19.6	19.6	19.5	20.1	19.2	18.3	19.2	19.4	18.4	18.7		73.8	71.13	73.44	71.94	73.02	71.76	73.81	70.04	64.76	66.39	74.25	71.72
21	19.4	19.4	19.6	19.7	19.7	19.6	19.9	19.6	19.6	20.1	20.4	19.2	19.7	20	19.4	19.6	20.2	17.9	20	19.4		71.39	73.37	72.31	73.78	71.57	73.68	69.66	64.39	65.89	74.34	72.03
22	18.8	18.8	19	18.6	19.6	19.6	19.2	19.6	19	19.6	19.3	18.8	18.6	18.4	19.6	21.8	20.8	18.7	18.7	19.2	19.4		72.7	81.52	70.95	69.63	70.73	69.1	64.27	65.65	70.54	74.54
23	19.1	19.6	20	19.5	19.9	19.3	20.2	19	19.9	19.8	20.4	18.9	19.2	19.8	20.4	18.9	19.2	19.7	19.9	19.1	19.2	20.3		73.2	72.46	70.86	72.22	69.96	63.98	65.42	72.14	73.49
24	18.3	18.8	18.3	17.8	18.1	18.1	17.7	18	18.1	18.6	18.5	18.4	18.6	17.9	18	19.1	19	18	18.1	17.9	18.6	24.1	19.4		71.32	69.81	71.12	69.37	63.66	64.98	71.0	75.29
25	19	18.9	19.2	18.9	19.8	19.6	19.5	19.6	19	19.9	18.6	18.7	19.1	18.6	19.6	19.4	19.1	18.1	19.3	18.9	19.2	19.8	19.1	19		72.19	73.59	70.2	64.33	66.66	74.62	71.28
26	19.7	18.9	19.7	18.7	19.3	19.4	19.1	19.5	18.9	19.5	18.9	18.5	19	19	18.7	19.2	19.1	19.3	19.5	18.8	19	20.4	19	18.1	19		72.15	69.02	65.03	66.83	72.11	70.48
27	19.5	19.4	20	19.7	20.1	20.1	20.1	19.7	19.8	20.5	19.9	19.8	19.8	19.6	19.4	20.3	19.7	17.2	19.7	19.5	19.8	20	19.6	18.6	19.7	19.1		69.72	64.35	67.37	75.0	71.36
28	18.2	18.1	19.1	18.6	18.3	17.8	18.1	18.1	18.5	21	18.4	17	18	17.9	19.7	19.5	19.2	18.5	18.8	17.2	19.2	18	19.2	18	18.8	18.7	17.8		63.63	64.77	69.61	69.97
29	16.9	19.4	18.1	18.9	20.6	19.8	20.1	19.9	20.5	20.1	17.9	18.7	20	17.6	20.6	19.7	20.2	19.9	19.2	20.8	19.5	17.9	18.8	19.4	20.8	19.8	19	19.4		65.03	64.72	63.91
30	17.5	17.6	18	19.8	17	16.9	16.9	16.6	17.5	17	17.4	16.7	16.4	16.2	20.7	17.5	17.3	19.2	17	17.5	15.7	21.4	18	17.1	17.8	16.6	19.4	18.4	19.4		66.85	65.87
31	20.8	20.9	20.9	19.1	19.7	19.6	19.7	19.8	18.8	20.2	19.1	19	19.4	19.2	18.8	19.8	22.5	17.9	20	19	19.6	20	19	19	19.3	19.4	19.9	18.3	19.6	16.2		71.06
32	18.3	18.9	18.8	17.9	19.3	18.9	19.4	20	19	19.6	19.4	18	18.2	19.1	18.5	19.3	18	18.1	18.6	18.2	19.4	19.4	21.4	19.4	19.2	18.1	18.8	18.3	19.4	20.8	18.3	

Taxa; 1- *Rhodobacter capsulatus* ATCC 11166^{T}; 2- *Rhodobacter viridis* JA737^{T}; 3- *Rhodobacter maris* JA276^{T}; 4- *Rhodobacter aestuarii* JA296^{T}; 5- *Rhodobacter sphaeroides* 2.4.1^{T}; 6- *Rhodobacter johrii* JA192^{T}; 7- *Rhodobacter megalophilus* JA194^{T}; 8- *Rhodobacter azotoformans* KA25^{T}, 9- *Rhodobacter ovatus* JA234^{T}; 10- *Rhodobacter vinaykumarii* JA123^{T}; 11- *Rhodobacter veldkampii* ATCC 35703^{T}; 12- *Rhodobacter blasticus* DSM 213^{T}, 13- *Cereibacter changlensis* JA139^{T}; 14- *Defluviimonas denitrificans* DSM 18921^{T}; 15- *Falsirhodobacter* sp. alg1; 16- *Haematobacter missouriensis* CCUG 52307^{T}; 17- *Paenirhodobacter enshiensis* DW2-9^{T}; 18- *Pseudorhodobacter ferrugineus* DSM 5888^{T}; 19- *Thioclava pacifica* DSM 10166^{T}; 20- *Gemmobacter aquatilis* DSM 3857^{T}; 21- *Pararhodobacter aggregans* DSM 18938^{T}; 22- *Rhodobaca barguzinensis* alga-05^{T}; 23- *Roseicitreum antarcticum* CGMCC 1.8894^{T}; 24- *Roseinatronobacter thiooxidans* DSM 13087^{T}; 25- *Rhodovulum sulfidophilum* DSM 1374^{T}; 26- *Albimonas donghaensis* DSM 17890^{T}; 27- *Paracoccus denitrificans* DSM 413^{T}; 28- *Roseobacter litoralis* Och 149^{T}; 29- *Escherichia coli* ATCC 11775^{T}; 30- *Pseudomonas aeruginosa* DSM 50071^{T}; 31- *Sinorhodobacter ferrireducens* CCTCC AB2012026^{T}; 32- *Roseibaca ekhonensis* CECT 7235^{T}. *Rba. sphaeroides* clade member values in red color; *Rba. capsulatus* clade member values in light brown color; *Rba. veldkampii* clade (*Rba. veldkampii* and *Rba. vinaykumarii*) member values in pink color.

Table 3.11 AAI and POCP values of genus *Rhodobacter* members and some related genera

AAI values

	Rba. capsulatus clade				*Rba. sphaeroides* clade					POCP values																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1		79.94	75.39	72.62	59.57	57.11	57.23	57.94	59.42	66.58	63.18	62.78	55.21	54.54	52.9	54.07	59.9	55.05	61.88	57.97	48.31	50.86	48.18	50.41	55.36	46.27	49.46	46.1	17.81	21.38	62.21	47.7
2	89.21		73.29	72.1	57.19	54.95	54.88	56.16	59.59	64.41	61.68	61.95	54.25	55.32	50.69	52.28	58.54	55.22	61.09	59.3	47.56	50.32	47.58	50.19	52.41	45.71	48.73	46.46	17.36	21.13	61.86	47.64
3	78.54	78.44		75.63	57.57	55.4	57.13	56.85	58.73	68.07	62.81	61.78	54.95	56.94	51.49	52.85	59.08	55.57	63.08	58.91	49.79	50.68	49.96	50.22	54.63	45.93	49.83	46.88	17.74	21.46	61.32	49.1
4	78.07	77.86	82.33		56.24	54.4	54.19	54.83	59.62	66.37	62.59	59.71	52.88	54.97	50.88	52.16	57.27	54.54	63.68	55.7	47.86	50.8	49.52	49.83	54	45.18	47.11	46.18	17.03	19.94	61.52	48.82
5	64.36	63.95	63.88	63.51		85.1	88.23	76.34	69.76	57.99	57.43	61.71	67.73	54.66	53.18	55.01	54.5	55.01	56.2	58.44	50.34	52.29	50.49	51.25	54.96	48.67	51.34	48.35	18.2	23.01	56.07	48.9
6	64.4	63.86	64.09	63.85	96.32		86.11	74.93	68.26	56.66	56.13	59.29	66.73	53.15	50.98	53.37	52.63	53.34	54.38	56.41	49.66	51.84	50.19	50.27	53.53	47.2	50.68	47.29	17.78	22.87	54.67	48.83
7	64.11	63.58	63.92	63.04	98.56	96.39		76.4	66.46	56.81	55.51	59.38	66.89	54.32	50.1	53.82	52.05	53.32	54.83	57.52	50.02	50.39	50.39	49.87	54.24	46.48	51.65	47.66	18.04	23.13	54.5	48.25
8	64.26	64.02	63.99	63.42	86.42	86.47	86.09		70.52	57.02	56.81	59.77	66.48	54.15	50.67	53.95	51.88	54.25	55.15	55.97	48.79	52.15	49.38	52.04	53.9	46.82	49.71	47.97	17.78	22.36	53.88	49.63
9	65.09	64.98	65.18	64.67	80.94	81.05	80.37	81.08		64.35	64.26	62.8	62.08	55.95	52.88	56.39	55.69	57.33	58.42	59.02	49.27	54.28	53.17	53.68	55.24	48.23	49.85	49.03	17.26	21.42	56.09	54.53
10	67.67	66.97	68.33	67.85	65.41	65.44	65.55	65.16	67.33		71.07	61.26	55.03	57.33	53.89	56.23	60.12	57.87	63.83	59.28	51.31	53.25	54.06	52.77	56.93	49.24	50.37	48.44	17.21	21.07	59.71	54.2
11	69.58	69.27	69.4	69.21	67.35	67.59	67.02	67.17	68.46	72.85		61.55	54.97	56.98	53.69	57.27	58.3	59.2	62.63	59.21	50.13	55.58	54.49	54.92	54.93	47.9	49.89	48.42	16.44	20.17	58.08	56.66
12	64.07	63.67	63.91	63.26	67.25	67	66.92	66.75	68	64.04	66.24		62.48	61.23	54.98	55.15	59.04	62.71	58.88	64.84	52.66	53.4	50.99	52.49	54.19	49.03	50.77	50.93	17.7	22.65	58.68	52.11
13	63.61	63.77	63.67	62.88	74.64	74.03	73.68	73.8	74.86	64.87	67.27	67.9		54.92	52.02	54.09	52.76	56.75	54.48	59.12	51.25	51.98	51.59	51.02	51.99	47.25	52.12	49.02	18.22	23.82	54.39	48.99
14	64.19	64.21	64.39	63.38	64.93	64.54	64.72	64.24	65.72	65.47	67.39	64.95	64.96		50.08	52.23	53.38	59.4	59.83	60.92	53.55	51.78	50.33	50.67	50.69	47.74	50.67	53.11	16.82	21.96	57.5	51.08
15	62.53	62.3	62.46	62.46	66.51	66.13	66.18	66.41	67.31	63.37	64.72	64.34	66.91	62.91		60.06	63.92	52.95	53	53.04	44.13	48.74	47.99	48.81	48.29	52.66	47.34	45.72	19.17	21.59	53.15	46.84
16	63.23	62.83	63.14	62.96	65.53	65.07	65.13	65.4	66.54	64.5	66.13	63.42	64.9	63.64	64.62		63.51	52.96	56.6	55.31	50.32	50.17	49.78	50.07	50.89	53.57	55.23	46.02	19.22	25.34	55.85	47.72
17	69.68	69.33	69.38	68.97	63.74	63.6	63.45	63.78	64.22	66.14	67.64	63.38	63.7	63.93	63.2	64.46		53.22	59.05	58.1	48.89	48.4	48.92	48.3	51.5	51.83	51.96	45.75	19.19	23.24	64.21	47.81
18	62.39	62.39	62.48	62.18	66.5	66.19	66.08	66.13	67.71	63.29	65.2	66.34	68.31	64.1	64.56	62.68	61.72		58.1	62.26	52.12	54.09	52.37	54.2	49.3	48.24	47.96	53.71	15.32	20.35	54.17	52.05
19	69.01	68.5	69.2	69.24	63.29	63.03	62.95	63.12	64.07	67.85	69.13	62.45	62.85	64.74	62.08	63.33	67.59	62.25		60.16	52.58	52.34	50.98	53.22	52.94	48.85	51.41	49.02	16.74	21.43	65.2	51.01
20	64.21	64.38	64.28	63.67	68.12	67.96	68.13	67.87	69.67	64.74	67.43	69.1	70.03	65.52	65.7	64.34	64.19	67.75	63.99		55.06	52.87	52.28	53.21	50.81	50.42	52.33	51.11	16.97	23.3	62.06	51.05
21	60.61	60.14	60.85	60.32	61.37	60.95	61.01	61.02	62.44	62.39	63.82	61.25	61.36	61.05	60.13	61.52	60.75	60.96	61.17	62		52.19	50.49	50.42	48.5	47.13	48.76	46.82	15.83	22.33	50.95	49.63
22	59.85	59.39	59.99	59.68	60.73	60.8	60.28	60.74	61.98	61.12	62.59	60.18	60.87	60.63	59.7	60.74	59.06	61.13	59.76	60.97	62		57.75	72.18	49.46	47.16	45.53	50.28	15.18	20.12	49.88	61.31
23	59.93	59.69	60.17	59.81	61.47	61.57	61.23	61.71	63.47	62.49	64.18	61.12	62.25	61.16	60.75	61.5	60.06	62.11	60.86	62.07	62.44	65.48		55.78	48.18	45.17	44.56	47.84	15.01	18.62	48.85	57.63
24	59.69	59.47	59.73	59.36	61.08	60.74	60.5	60.79	61.65	60.95	62.64	60.47	61.06	60.92	59.95	60.32	59.27	61.42	60.07	61.82	62.2	84.78	65.98		49.26	47.24	44.63	49.54	14.97	19.84	49.45	59.85
25	62.21	61.47	62.12	61.75	63.64	63.37	63.28	63.46	64.42	64.4	64.85	62.49	63.08	61.05	61.31	63	62.14	61.22	61.97	62.43	59.87	59.88	65.98	60.2		46.83	46.82	47.36	17.35	21.42	52.92	47.81
26	56.75	56.39	56.55	56.55	57.69	57.52	57.41	57.56	58.5	58.31	58.23	57.24	57.43	57.46	57.04	57.38	56.64	56.75	57.22	57.97	56.09	56.15	56.38	56.66	57.41		44.03	47.07	17.82	24.31	48.59	44.47
27	61.81	61.58	61.75	60.83	61.69	61.5	61.5	61.76	62.88	63.45	64.53	60.69	61.4	61.37	61.53	63.24	63.39	59.63	61.86	61.47	58.65	57.89	58.43	58.07	59.2	55.87		44.3	18.87	25.49	52.66	43.5
28	58.49	58.85	58.6	58.46	59.19	58.89	58.79	59.18	60.31	59.82	60.99	59.52	59.96	60.47	59.56	59.38	58.64	61.22	59.09	60.42	57.7	59.18	59.11	59.61	60.25	56.38	56.41		14.53	19.69	47.64	48.27
29	40.23	39.88	39.78	39.7	40.25	40.29	40.06	39.95	39.98	40.19	39.89	40.01	40.27	39.7	40.84	41.04	41.02	39.14	39.67	39.82	39.23	39.23	38.94	38.85	39.79	40.42	40.33	39.04		30.64	17.9	14.32
30	42.18	41.91	42.12	41.48	42.3	42.36	42.25	42.18	42.27	42.22	42.1	42.22	42.57	41.74	42.13	43.41	42.56	41.37	41.57	42.5	41.66	41.28	41.33	41.39	41.89	42.84	43.14	40.58	48.07		23.54	18.4
31	72.13	71.9	72.17	71.42	63.91	63.85	63.6	63.51	64.44	67.18	69.55	63.37	63.63	64.68	63.36	64.06	73.59	61.95	70.57	65.02	60.32	59.02	60.1	59.81	62.56	56.81	62.72	58.62	40.09	42.42		48.06
32	59.13	58.79	58.98	58.75	59.99	60.15	59.48	60.15	61.81	61.1	62.45	60.05	59.95	59.87	59.67	59.84	59.28	60.78	59.58	60.28	61.28	71.67	65.53	71.35	59.67	56.15	57.54	58.96	38.62	41.06	59.11	

Taxa; 1- *Rhodobacter capsulatus* ATCC 11166^{T}; 2- *Rhodobacter viridis* JA737^{T}; 3- *Rhodobacter maris* JA276^{T}; 4- *Rhodobacter aestuarii* JA296^{T}; 5- *Rhodobacter sphaeroides* 2.4.1^{T}; 6- *Rhodobacter johrii* JA192^{T}; 7- *Rhodobacter megalophilus* JA194^{T}; 8- *Rhodobacter azotoformans* KA25^{T}, 9- *Rhodobacter ovatus* JA234^{T}; 10- *Rhodobacter vinaykumarii* JA123^{T}; 11- *Rhodobacter veldkampii* ATCC 35703^{T}; 12- *Rhodobacter blasticus* DSM 213^{T}, 13- *Cereibacter changlensis* JA139^{T}; 14- *Defluviimonas denitrificans* DSM 18921^{T}; 15- *Falsirhodobacter* sp. alg1; 16- *Haematobacter missouriensis* CCUG 52307^{T}; 17- *Paenirhodobacter enshiensis* DW2-9^{T}; 18- *Pseudorhodobacter ferrugineus* DSM 5888^{T}; 19- *Thioclava pacifica* DSM 10166^{T}; 20- *Gemmobacter aquatilis* DSM 3857^{T}; 21- *Pararhodobacter aggregans* DSM 18938^{T}; 22- *Rhodobaca barguzinensis* alga-05^{T}; 23- *Roseicitreum antarcticum* CGMCC 1.8894^{T}; 24- *Roseinatronobacter thiooxidans* DSM 13087^{T}; 25- *Rhodovulum sulfidophilum* DSM 1374^{T}; 26- *Albimonas donghaensis* DSM 17890^{T}; 27- *Paracoccus denitrificans* DSM 413^{T}; 28- *Roseobacter litoralis* Och 149^{T}; 29- *Escherichia coli* ATCC 11775^{T}; 30- *Pseudomonas aeruginosa* DSM 50071^{T}; 31- *Sinorhodobacter ferrireducens* CCTCC AB2012026^{T}; 32- *Roseibaca ekhonensis* CECT 7235^{T}. *Rba. sphaeroides* clade member values in red color; *Rba. capsulatus* clade member values in light brown color; *Rba. veldkampii* clade (*Rba. veldkampii* and *Rba. vinaykumarii*) member values in pink color.

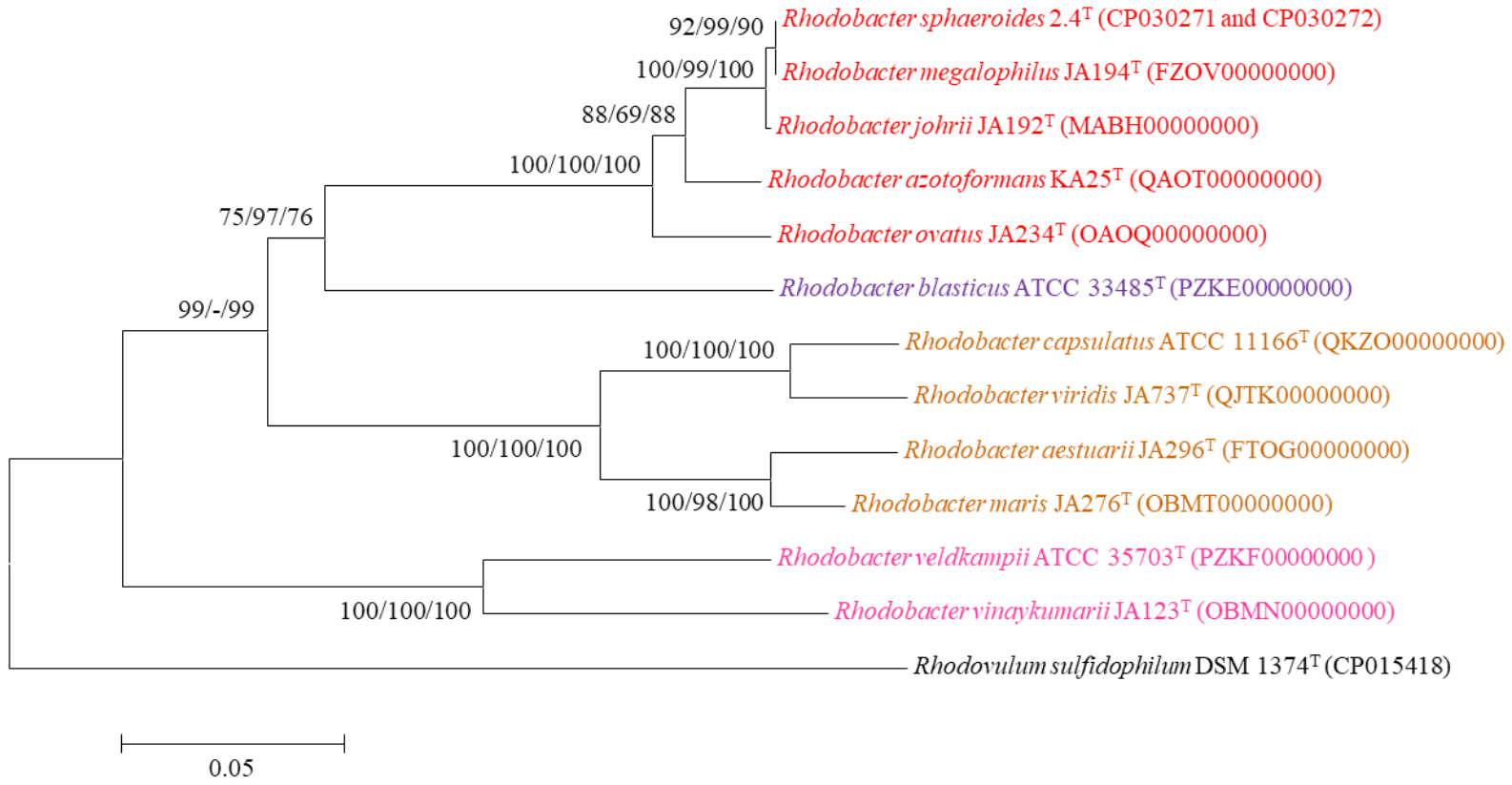

Fig. 3.22 Phylogenetic analysis PufL and M concatenated protein sequences; tree was constructed using MEGA7 software. The evolutionary distances were computed using the poisson correction method. The bootstrap values are calculated by 1000 replicates and the values are representing the bootstrap values of NJ/ML/ME tree.

3.3.2.6.2 Photosynthetic reaction center protein (PufX)

Crouch and Jones (2012) showed that some of the current members of the genus *Rhodobacter* (*Rba. sphaeroides*, *Rba. azotoformans*, *Rba. Blasticus*) and *C. changlensis* (formerly *Rba. changlensis*) form RC-LH1 (photosynthesis reaction center-light-harvesting 1) complex dimers instead of monomers, as in *Rba. capsulatus*, *Rba. veldkampii* and *Rba*. *vinaykumarii*. The dimer formation is facilitated by the PufX protein, by interacting with two monomers of RC–LH1 complex, without disturbing their structure (Crouch and Jones, 2012). Deletion of the *pufX* gene in *Rba. sphaeroides* resulted in the formation of only RC-LH1 monomers and loss of the ability to grow phototrophically. However, in contrast, *Rba. veldkampii* in spite of having the PufX coding gene, forms only monomers and can grow phototrophically. This result indicated that RC-LH1 dimer formation is not only dependent on the presence or absence of PufX protein but also on some unidentified factors (Crouch and Jones, 2012). Mutational studies on PufX protein C-terminal sequences of *Rba. sphaeroides* reveal the importance of R49, G52 or R53 amino acids and alteration of these sequences prevents RC-LH1dimer formation (Qian et al., 2017).

To analyse members of *Rhodobacter* spp. for the presence of the sequence pattern required for RC-LH1 dimer formation, we extracted the PufX protein sequences of *Rhodobacter* members from NCBI and aligned them using CLUSTALW. Interestingly, all the members of *Rba. sphaeroides* clade have R49, G52 or R53 amino acids (Fig. 3.23), while none of the other members of *Rhodobacter* outside of the *Rba. sphaeroides* clade has such a pattern. A phylogenetic tree (Fig. 3.24) constructed using the PufX protein sequences of *Rhodobacter* members and some phylogenetically related chemotrophic bacteria shows that *Rhodobacter* spp. are polyphyletic.

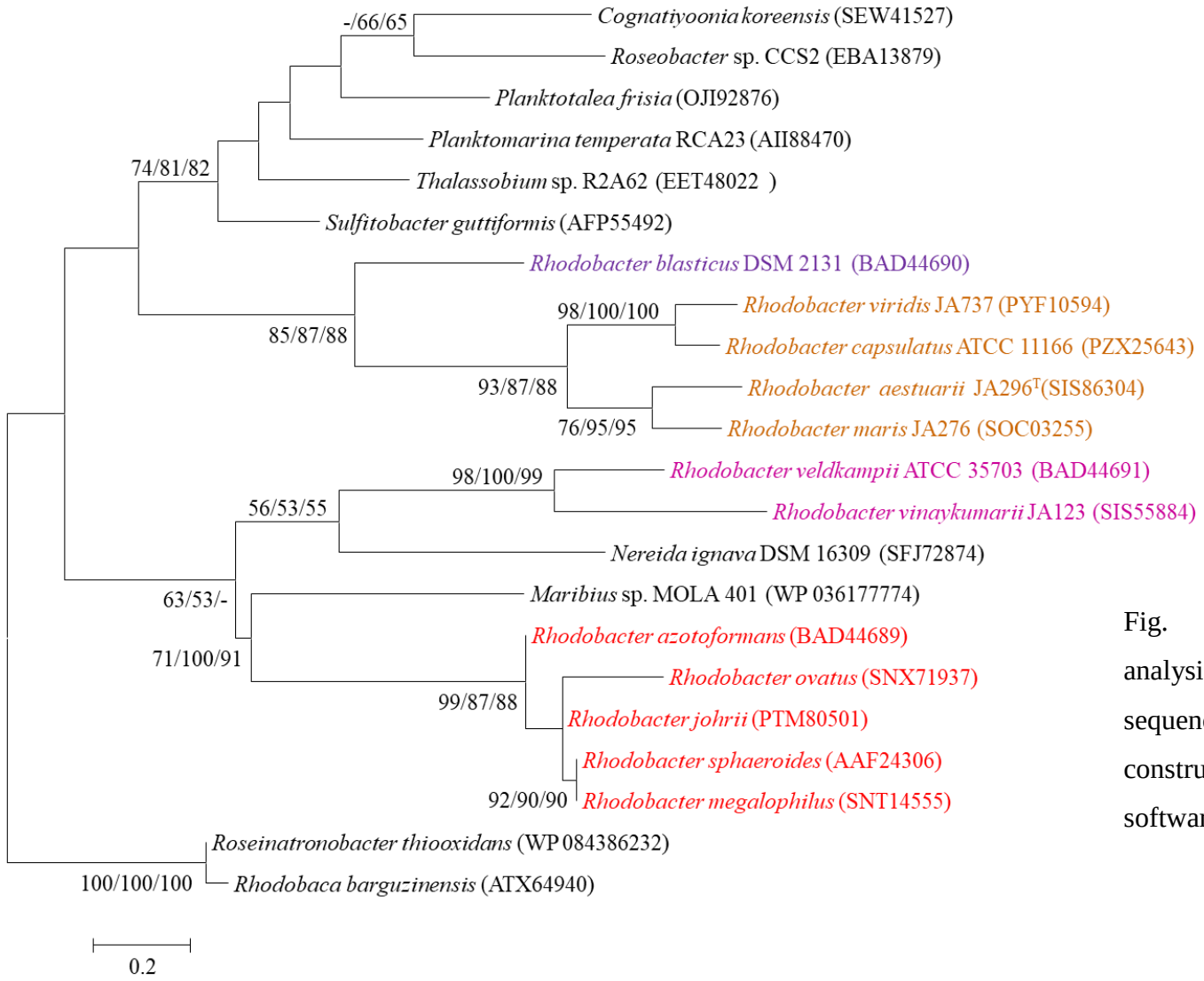

Fig. 3.23 Phylogenetic analysis of pufX protein sequences, tree was constructed using MEGA7 software

Fig. 3.24 PufX protein sequence alignment using clustalW, showing the R49, G52 or R53 sequence pattern, which is important for RC-LH1 dimer formation.Taxa. 1, *Rba. sphaeroides* ATH 2.4.1 (AAF24306); 2, *Rba. megalophilus* JA194 (SNT14555); 3, *Rba. johrii* JA192 (PTM80501); 4, *Rba. azotoformans* KA25(BAD44689); 5, *Rba. ovatus* JA234 (SNX71937); 6, *Rba. veldkampii* ATCC 35703(BAD44691); 7, *Rba. vinaykumarii* JA123 (SIS55884); 8, *Rba. viridis* JA737 (PYF10594); 9, *Rba. capsulatus* (PZX25643); 10, *Rba. aestuarii* JA296 (SIS86304); 11, *Rba. maris* JA276 (SOC03255); 12, *Rba. blasticus* ATCC 33485 (BAD44690). The sequence pattern of R49, G52 or R53 (red color) was seen in the *Rba. sphaeroides* clade.

3.3.2.6.3 Photosynthetic gene cluster (PGC)

All essential genes required for photosynthesis are organized in a continuous stretch called the photosynthetic gene cluster (PGC). Phylogenetic analysis of one or two photosynthetic genes (Imhoff et al., 2018) is not sufficient to study the photosynthetic gene phylogeny among the members of the 'Rhodobacteraceae' (Brinkmann et al., 2018). Thus, in addition to the PufLM or PufX protein based phylogenetic analysis, we studied the photosynthetic gene arrangement in the PGC to differentiate *Rhodobacter* species. Comparative analysis of the PGC of *Rhodobacter* spp. (Fig. 3.25) revealed that members of the *Rba. sphaeroides* clade lack the *pufB* gene sequence encoding for light-harvesting antenna LH1-beta subunit. In contrast the *acsF* gene encoding for the Mg-protoporphyrin IX oxidative cyclase, aerobic form, was not found in the genomes of members of the *Rba. capsulatus* clade (Fig. 3.25). In the *Rba. sphaeroides* clade, urea metabolism genes were seen after the *cycA gene*, whereas in the remaining clades these genes are scattered elsewhere in their genomes but not adjacent to the PGC. The PGC gene encoded protein sequences were concatenated and used to construct a phylogenetic tree (Fig. 3.26), which was congruent with the PufLM protein, PufX protein and 16S rRNA gene phylogenetic trees. In the PGC based phylogenetic tree *Rhodobacter* spp. formed four monophyletic clades (Fig. 3.26). The phylogenomic trees constructed using protein sequences (Fig. 3.27; Fig. 3.28) are also in in congruence with the UBCG based, PGC based phylogenetic trees.

3.3.2.7 Polar lipid analysis

The polar lipid composition of *Rhodobacter* spp. typically includes phosphatidylethanolamine, phosphatidylglycerol, uncharacterised aminolipid,

phosphatidylcholine (except *Rba. veldkampii*) and other unidentified lipids. In addition, clade I members have DPG and an unidentified glycolipid (GL). Polar lipid profiles of the representative strains are shown in Fig. 3.29. Along with polar lipid other phenotypic and chemotaxonomic characters between the *Rhodobacter* clades and related genera was given in the Table 3.12.

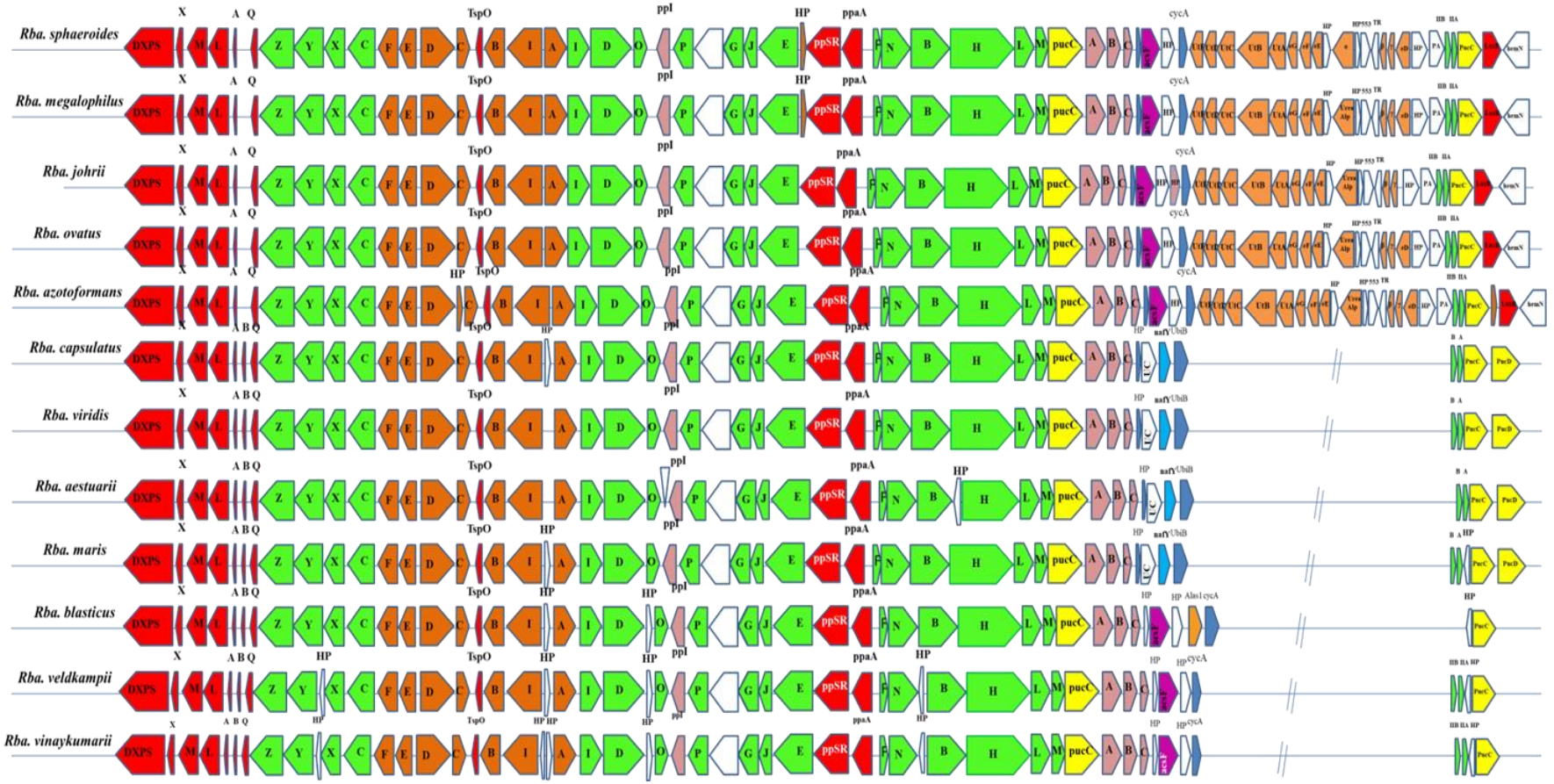

Fig. 3.25 Arrangement of photosynthetic gene cluster and structure in *Rhodobacter*. Green , bacterial chlorophyll genes; red, *puf* and regulators genes; pink , *puh* genes; orange , carotenoid genes; blue , *hem* and *cyc* gene; yellow, *lha*A gene; blank , uncertain or unrelated genes; grey, hypothetical protein.

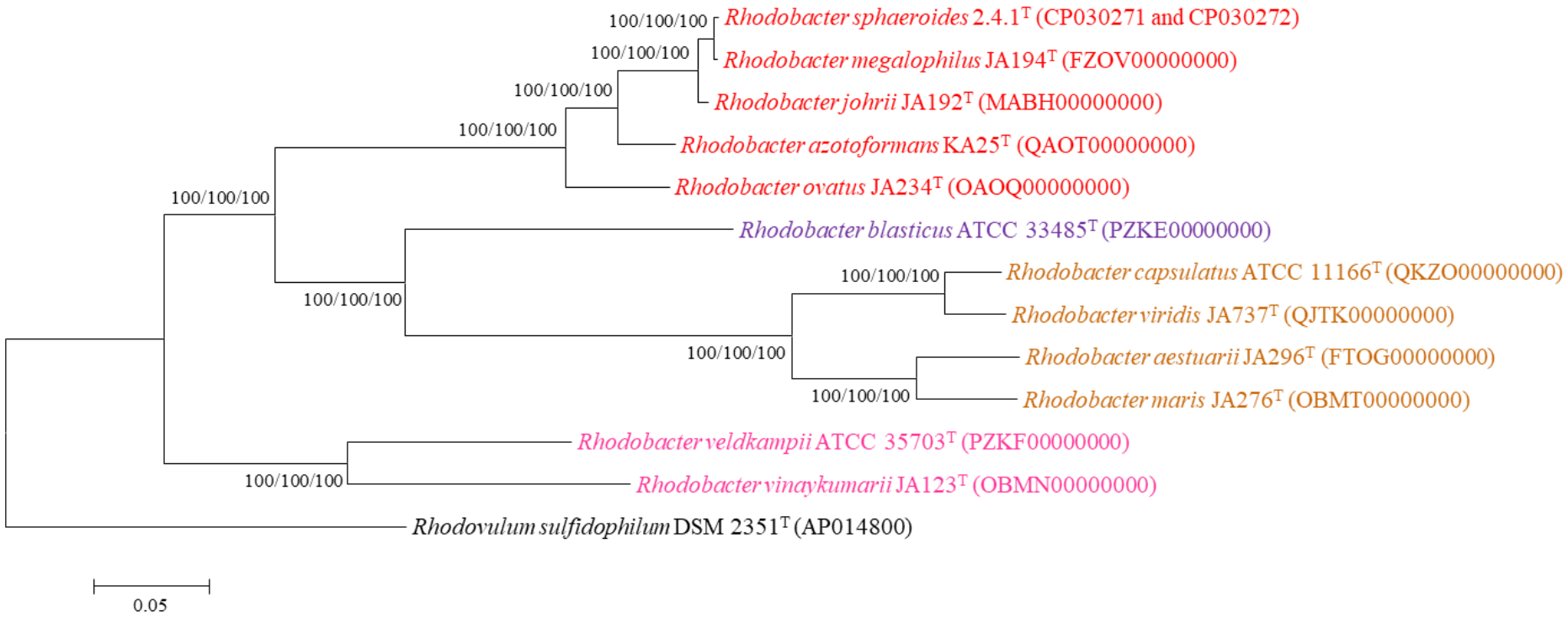

Fig. 3.26 PGC Phylogenetic analysis of PGC (Photosynthetic gene cluster) concatenated protein sequences; tree was constructed using MEGA7 software. The evolutionary distances were computed using the poisson correction method. The bootstrap values are calculated by 1000 replicates and the values are representing the bootstrap values of NJ/ML/ME tree.

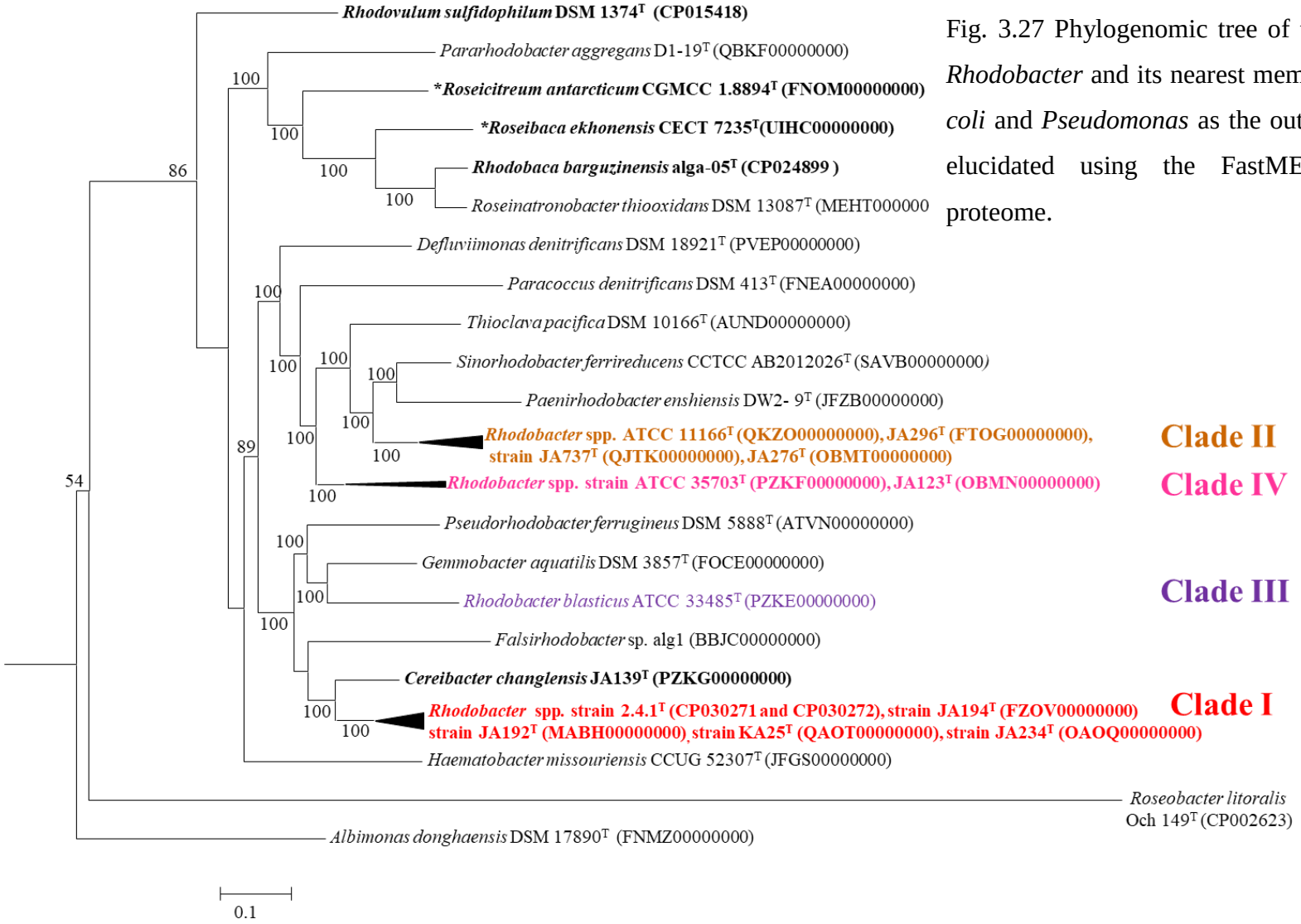

Fig. 3.27 Phylogenomic tree of type strains of genus *Rhodobacter* and its nearest member with *Escherichia coli* and *Pseudomonas* as the out group. The tree was elucidated using the FastME from the whole proteome.

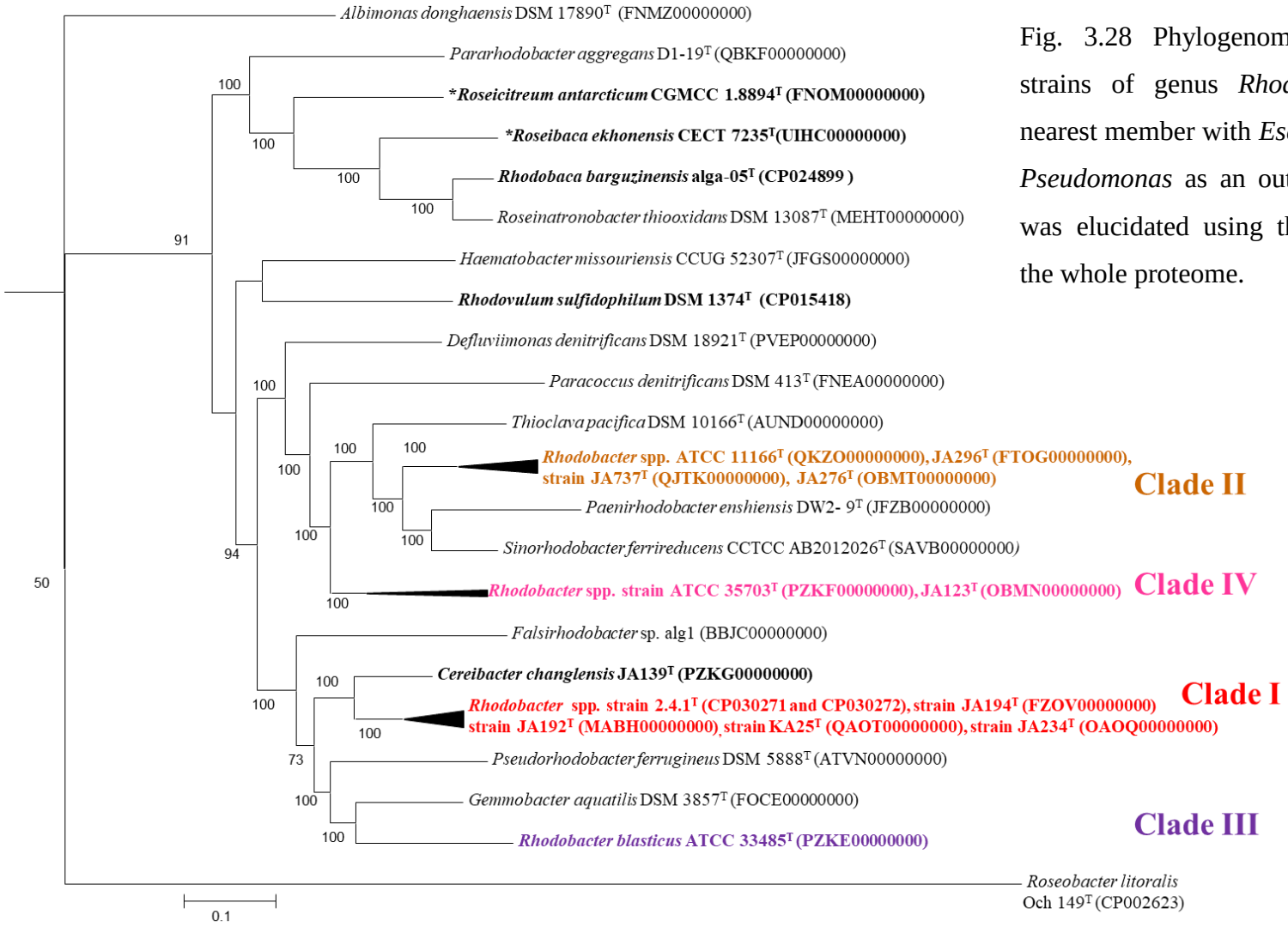

Fig. 3.28 Phylogenomic tree of type strains of genus *Rhodobacter* and its nearest member with *Escherichia coli* and *Pseudomonas* as an out group. The tree was elucidated using the RAxML from the whole proteome.

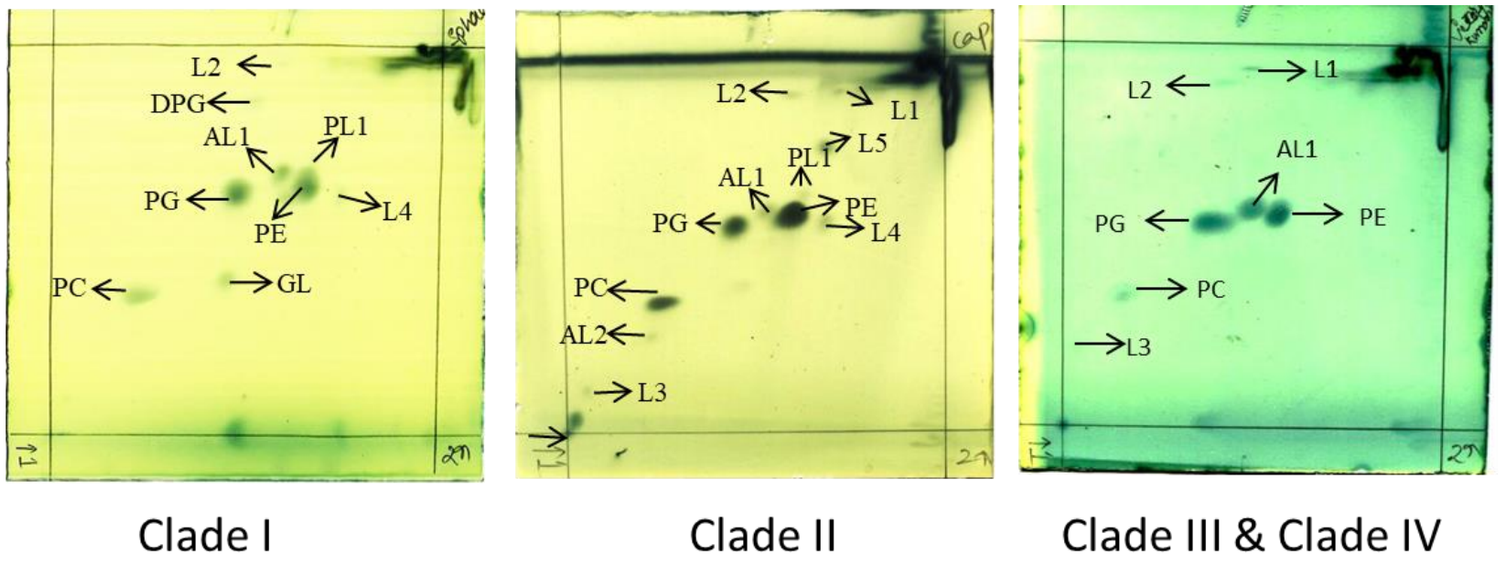

Fig. 3.29 Two-dimensional thin-layer chromatogram of polar lipids profile of different clades of *Rhodobacter* spp. clade I (*Rba. sphaeroides Rba. johrii, Rba. megalophilus, Rba. azotoformans*, *Rba. ovatus*, *Rba. alkalitolerans*), clade II (*Rba. capsulatus* , *Rba. viridis*, *Rba. azollae*, *Rba. sediminis*. *Rba. aestuarii*, *Rba. maris, Rba. lacus*), clade III (*Rba. blasticus*); clade IV (*Rba. veldkampii* clade having *Rba. veldkampii* and *Rba. vinaykumarii*); PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PL1,2, uncharacterized phospholipids; AL1,2, uncharacterized aminolipids; GL, Glycolipid; APL, aminophospholipid; L1,2,3,4,5 uncharacterized lipids.

Table 3.12 Differentiating characters of taxa of the genus *Rhodobacter* and closely related genera of the family 'Rhodobacteraceae'.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cell shape	R-O	R-O	R-O	R-O	R	R-O	R	R-O	R	R,O	R	R-O	R	C-R
Motility	+/-	+	-	-	-	-	+/-	+/-	-	+/-	-	+/-	+/-	+/-
Phototrophic growth	+	+	+	+	+	+	-	-	-	-	-	+	-	-
Multiplication	Binary fission	Binary fission	Sessile budding	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission/ Budding	nd	nd	Binary fission	Binary fission	nd	Binary fission
Slime production	+/-	+/-	-	-	+	+	-	Nd	nd	nd	nd	+/-		nd
Growth at 5^0C	+/-	-	-	-	-	+	-	+/-	-	-	+	nd	-	+/-
Vitamins	b,n,t	b,n,t	n,t,b	b,paba,t	b	b,n,t	-	b,n,t,paba,B_3,B_{12}	nd	nd	b,n,t,paba,B_3	b,n,t,paba, B_{12}, ć	B_3,B_{12}	nd
NaCl requirment	-	-	-	-	+	-	+	+/-	+	+	-	+/-	-	+
Sulfate assimilation	+	+	+	-	+	+	nd	Nd	nd	nd	nd	+/-	-	-
Denitrification	+/-	-	-	-	-	-	-	+/-	-	+/-*	-	-	-	+/-
BChl *a*	+	+	+	+	+	+	-	-	-	-	-	+	-	-
IMA	Vesicles	Vesicles	Lamellar	Vesicles	Vesicles	Vesicles	-	-	-	-	-	Vesicles	-	-
Production of carotenoids	+	+	+	+	+	+	-	-	-	-	-	+	-	-

Light harvesting complexes	LH1,LH2	LH1,LH2	LH1,LH2	LH1,LH2	LH1,LH2	LH1,LH2	-	-	-	-	-	LH1,LH2	-	-
Photoautotrophic growth	+/-	+/-	+	+	-	-	-	-	-	-	-	+/-	-	-
Dark anaerobic growth	+/-	+/-	-	Nd	-	+	-	-	-	-	-	+/-	-	-
Fatty acids			Nd											
$C_{18:0}$3OH	-	+		-	-	-	-	+	-	+/-	-	-	-	+/-
$C_{18:1}\omega$7c11 methyl	+	-		-	+	+	+/-	+/-	-	+/-	-	+	+	+/-
Polar lipids														
GL	+	-	-	-	-	+	+	+/--	-	-	-	-		+
SL	-	-	-	-	-	-	-	-	-	-	-	+	nd	-
PC	+	+	+	-	+	+	+	+	-	+	-	-		+
DPG	+	-	-	-	-	+	+	+/-	-	-	-	-		+
Isolation source	Fresh, soil	Fresh and Marine	Freshwater	Freshwater	Marine	Snow sample	Soil, Slattern	Freshwater	Blood, Nose	Marine	Soil	Hyper-saline and marine	Marine	Soil, sewage

1, *Rhodobacter sphaeroides* clade (*Rba. sphaeroides, Rba. johrii, Rba. megalophilius, Rba. azotoformans, Rba. ovatus* and *Rba. alkalitolerans*; data from this study, Gandham et al., 2018; Girija et al., 2010; Arunasri et al., 2008; Srinivas et al., 2008; Hirashi et al.,1996; Imhoff., 2005); 2, *Rhodobacter sensu stricto* clade (*Rba. capsulatus, Rba. aestuarii, Rba. maris, Rba. viridis, Rba. sediminis, Rba. lacus* and *Rba. azollae*; data from this study and from Subhash and Lee., 2016; Shalem Raj et al., 2013; Girija et al., 2010, Venkata Ramana et al., 2009, Anil Kumar et al., 2009, Imhoff., 2005; Suresh et al., 2017); 3, *Rhodobacter blasticus* clade (*Rba. blasticus*; data from this study, data taken from Eckersley and Dow, 1980; Girija et al., 2010); 4, *Rhodobacter veldkampii* clade (*Rba. veldkampii;* data from this study, data taken from Hansen and Imhoff, 1985; Girija et al., 2010); 5, *Rba. vinaykumarii* clade (*Rba. vinaykumarii*; data from this study, data taken from Srinivas et al., 2007; Girija et al., 2010; 6, *Cereibacter* (*Cer. changlensis*; data taken from Suresh et al., 2015, Girija et al., 2010); 7, *Falsirhodobacter* (*F. deserti, F. halotolerans*; data taken from Wang et al., 2015; Subhash et al., 2013]; 8, *Gemmobacter* (ten species; data taken from Suresh et al., 2015; Sheu et al., 2013; Chen et al., 2013a; Rothe et al., 1987; Kämpfer et al., 2015; Liu et al., 2014); 9, *Haematobacter* (*H. missouriensis, H. massiliensis*; data taken from Helsel et al., 2007; Subhash et al., 2013; Wang et al., 2014); 10, *Pseudorhodobacter* (four strains, data taken from Chen et al., 2013b; Lee et al., 2013; Jung et al., 2012; Uchino et al., 2002; Zhang et al., 2016); 11, *Paenirhodobacter* (*P. enshiensis*; data taken from Wang et al., 2014]; 12, *Rhodovulum* (based on nineteen species including the recently described *Rdv. aestuarii*; data taken from Suresh et al.,2015; Lakshmi et al., 2011; Srinivas et al., 2012, 2014; Nupur et al., 2014; Divyasree et al., 2015); 13, *Pararhodobacter* (*P. aggregans*; data taken from Xie., et al., 2015; Foesel et al., 2011); 14, *Paracocus* (based on forty one species; data taken from Kampfer et al.,2012; Zhang et al., 2014; Pan et al., 2014; Sun et al., 2013). R-O, rod to oval; R, rod; C-R, coccoid to rod; b, Biotin; B_{12}, vitamin B_{12}; B_3, vitamin B_3; n, niacin; paba, p-aminobenzoic acid; t, thiamine; ć , Complex requirement; PC, Phosphatidylcholine; SL, Sulfolipid; GL, Glycolipid; DPG, Diphosphatidylglycerol; LH, light harvesting system; +, present; -, absent; *,data of nitrate reduction; nd, No data available; IMA, Internal membrane architecture.

Discussion

4. Discussion

4.1 Validation of *Rhodobacter* spp. names (isolated during the study)

The genus *Rhodobacter* (*Rba*.) proposed by Imhoff et al. (1984) accommodated a few species of purple non sulfur anoxygenic phototrophic bacteria namely, *Rba. capsulatus* (type species), *Rba. sphaeroides, Rba. sulfidophilus* and *Rba. adriaticus*. However, *Rba. sulfidophilus* and *Rba. adriaticus* were subsequently reclassified as *Rhodovulum sulfidophilum* and *Rhodovulum adriaticum*, respectively (Hiraishi and Ueda, 1994). At the time of starting this work, the genus *Rhodobacter* comprised of 12 validly published species names; *Rba. aestuarii, Rba. azotoformans, Rba. blasticus, Rba. capsulatus, Rba. johrii, Rba. maris, Rba. megalophilus, Rba. ovatus, Rba. sphaeroides, Rba. veldkampii, Rba. vinaykumarii* and *Rba. viridis*. four more species ie., *Rba. lacus* (Suresh et al., 2017), *Rba. sediminis* (Subhash and Lee, 2016), *Rba. thermarum* (Khan et al., 2019) and *Rba. flagellates* (Xian et al., 2019) were subsequently added to the genus *Rhodobacter*. As a part of this study three new species (represented by *Rhodobacter* sp. JA912^T, *Rhodobacter* sp. JA916^T and *Rhodobacter* sp. JA983^T) belonging to the genus *Rhodobacter* were described and their names validated.

4.1.1 *Rhodobacter sp*. JA912

Even though the 16S rRNA gene sequence identity was more than 98.7%, low hybridization values (less than 30%) were observed when strain JA912^T was hybridized with its closest phylogenetic neighbours (Fig. 3.6), indicating that the strain JA912^T represents a new species of the genus *Rhodobacter*, according to the recommendations for delineating a bacterial species (Stackebrandt and Goebel, 1994; Wayne et al., 1987). While, all phylogenetically related members have phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC)

and an aminolipid (AL1) (Fig. 3.5) as major/minor polar lipids, the strain JA912^T can be differentiated from existing species by the presence of an unidentified amino lipid (AL2), two unidentified phosopholipids (PL1,2) and six unidentified lipids (L2-7). Together based on genomic (low DDH value) and chemotaxonomic (polar lipid profile) differences the strain represents a new species of the genus *Rhodobacter*, for which we propose the name *Rhodobacter azollae* sp. nov.

4.1.1.1 Description of *Rhodobacter azollae* sp. nov.

(*a.zol'lae*. N.L. fem. gen. n. *azollae* of the water fern *Azolla*)

Cells are ovals to rods (1.0-1.5 x 1.0-3.0 µm) that multiply by binary fission and have vesicular internal membrane structures, motile by single polar flagellum. Color of photosynthetically grown culture is yellowish brown. *In vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 377, 476, 509, 595, 800 and 857 nm. Bacteriochlorophyll-*a* and spheroidene are the photosynthetic pigments. Q-10 is the major quinone. Optimum growth occurs at 30 ºC (range 25–35 ºC) and at pH 7.0 (range pH 6.0–8.0). NaCl is not obligatory for growth, can tolerate up to 2 %. Photoorganoheterotrophy with a few organic compounds is the preferred mode of growth. Growth is observed with pyruvate, malate, acetate, succinate, fumarate, glucose, fructose, mannitol, glycerol, methanol, peptone, casamino acids, sucrose and aspartate. Photolithoautotrophy, chemolithoautotrophy and fermentative growth were not observed. Ammonium chloride, glutamate, aspartate and molecular nitrogen are good nitrogen sources. Biotin and thiamine were required as growth factors. $C_{18:1}\omega7c/C_{18:1}\omega6c$ is the major fatty acid. Minor amounts of $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{10:0}3OH$, $C_{18:1}\omega5c$, $C_{18:0}3OH$, $C_{16:0}$, $C_{18:0}$ and $C_{17:0}$ are present. Phosphatidylglycerol, phosphatidylethanolamine, phosphotidylcholine, two unidentified aminolipids

(AL1,2), two unidentified phospholipids (PL1,2) and seven unidentified lipids (L1-7) as the polar lipid composition. DNA base composition is 64.6 mol% G+C (HPLC).

Type strain JA912^T (=KCTC 15475^T =LMG 28748^T) was isolated from *Azolla filiculoides* of a fresh water pond in Gujarat, India. The genome sequencing study of strain JA912^T is being taken up and will be completed by end of 2020.

4.1.2 *Rhodobacter sp.* JA916

The EzBioCloud search analysis of 16S rRNA gene sequence of strain JA916^T indicated that it might be a new species belonging to genus *Rhodobacter* as the identity was less than 98.7%. Both the 16S rRNA (Fig. 3.4) and *rpoB* (Fig. 3.8) gene based phylogenetic trees showed that the strain forms a separate sub-clade within the clade formed by its phylogenetic neighbours, supporting the proposal of strain JA916^T as a new species. Further low (< 40%) DNA-DNA hybridization values between strains JA916^T with phylogenetically related type strains confirm the eligibility of strain JA916^T as a new species.

Strain JA916^T resembles the type strains of its phylogenetically closest neighbors with respect to phototrophic growth, presence of BChl *a* and carotenoids, vesicular ICM architecture, presence of $C_{18:1}\omega7c/C_{18:1}\omega6c$ and $C_{18:0}$ as major fatty acids and DPG, PG, PE, GL and PC as major polar lipids. However, strain JA916^T differs from its respective type strains with regard to growth at pH 10, photolithoautotrophy, organic substrate utilization (Table 3.3), presence of $C_{15:0}$-2OH and absence of $C_{17:0}$ fatty acids (Table 3.3).

The phenotypic differences are supported by the molecular differences (16s rRNA and *rpoB* gene phylogenetic analysis, low DDH values) warranted the

description of strain JA916^T into a new species of the genus *Rhodobacter* for which we propose the name *Rhodobacter alkalitolerans* sp. nov.

4.1.2.1 Description of *Rhodobacter alkalitolerans* sp. nov.

alkalitolerans (al.ka.li.to'le.rans. N.L. n. *alkali*, alkali; L. part. adj. *tolerans*, tolerating; N.L. part. adj. *alkalitolerans*, alkali-tolerating).

Color of phototrophically grown culture is yellowish brown. The *in vivo* absorption spectrum shown maxima at 377, 476, 509, 595, 800 and 857 nm. No requirement of NaCl for growth, but tolerance up to 3% NaCl. Optimum pH and temperature for growth are 7.0 (range 6.0-10) and 30 ºC (range 25-35 ºC), respectively. Acetate, ascorbate, cysteine, ethanol, fructose, fumarate, glucose, glycerol, lactate, malate, mannitol, pyruvate, succinate, sucrose, tartrate as carbon sources/electron donors support the growth of the strain JA916^T. Ammonium chloride, glutamine and glutamate are used as nitrogen sources for growth. Require biotin and thiamin for growth. Fatty acid $C_{15:0}$2OH is present in significant quantities. Q-10 is the respiratory quinone. The polar lipids consist of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, two unidentified phospholipids, unidentified aminolipids, unidentified glycolipids and four unidentified lipids. The DNA G+C content of the type strain is 65.1 mol% (by HPLC).

The type strain JA916^T (=KCTC 15473^T = LMG 28749^T) was isolated from an alkaline brown pond of Gujarat, India. The genome sequence of strain JA916^T will be carried out shortly.

4.1.3 *Rhodobacter sp*. JA983

16S rRNA gene based phylogenetic analysis and tree clustering analysis (Fig. 3.4) showed affiliation of strain JA983^T to the genus *Rhodobacter*. However, differences in fatty acid composition (presence of $C_{18:0}\omega5c$, $C_{15:0}2OH$ and absence of *iso*-$C_{16:0}$ and $C_{19:0}cyclo\omega10c/C_{19:0}\omega6c$), polar lipid profile (presence of AL2, L1 and L2 and absence of L3 and phospholipid 1), organic carbon utilization (inability to utilize glucose and fructose), low genomic size (Table 3.4) compared to its phylogenetic neighbors revealed that strain JA983^T represents a new species of genus *Rhodobacter*. Along with phenotypic characters, low OrthoANI (85-86%) and *d*DDH (30–45%) values with its phylogenetic neighbors necessitate the proposal of strain JA983^T as a new species of the genus for which we propose the name *Rhodobacter sediminicola* sp. nov.

4.1.3.1 Description of *Rhodobacter sediminicola* sp. nov.

(se.di.mi.ni'co.la. L. neut. N. *sedimen* sediment; L. suff. *–cola* (from L. masc. or fem. N. *incola*) inhabitant, dweller; N.L. masc. n. *sediminicola* dweller of sediments)

The color of phototrophically grown culture is yellowish brown but it was reddish brown under the chemotrophic condition. The colony was round shaped, reddish brown in color in the center, in the periphery white color can be seen (when it was grown in the plate/ aerobic condition). But anaerobically grown colonies are yellowish brown in color without white color in the periphery. Cells are rod to oval in shape, multiplied by binary fission and have polar flagellum for motility. Intact cells of strain JA983^T showed absorption maxima at 385, 449, 477, 510, 589, 810 and 851nm. NaCl was not required for growth but can tolerate up to 5%. Optimum pH and temperature for growth are 7.0 (range 6.0-8.0) and 30 ºC (range 25-35 ºC), respectively. Strain could grow by photolithoautotrophic, photoorganoheterotrophic,

chemoorganoheterotrophic and fermentative growth modes but not chemolithoautotrophic growth mode. Acetate, 2-oxoglutarate, malate, mannitol, glutamate, glycerol, pyruvate, sorbitol, cysteine, formate, propionate, tartrate and ethanol as carbon sources/electron donors support growth. Ammonium chloride, glutamine and glutamate are used as nitrogen sources for growth but nitrate, nitrite and urea did not support growth. Sulfide, cysteine, methionine, sulfate and thiosulfate were utilized as sulfur sources by the strain but not sulfite or elemental sulfur. Niacin and thiamin are required for growth. $C_{18:1}\omega7c/C_{18:1}\omega6c$ is the major fatty acid. Q-10 is the respiratory quinone. Phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylcholine, two unidentified aminolipids, one unidentified glycolipid and two unidentified lipids are the polar lipids. The DNA G+C content of the type strain is 68.4 mol% (calculated from the genome).

The type strain JA983^T (=KCTC 15782^T =NBRC 113843^T) was isolated from sediment of a freshwater pond of Gujarat, India.The GenBank accession number for the 16S rRNA gene sequence of *Rhodobacter sediminicola* JA983^T is LR596790 and the draft genome has been deposited in GenBank under the accession number VDEK000000000.

4.2 Reclassification of *Rhodobacter* spp.

Taxonomy and nomenclature are dynamic because of new approaches towards taxonomy and ever increasing amount of data (Dobler and Braveny, 2003). Mainly, taxonomic errors are due to the combination of phenotypic based classification and poor resolution of 16S rRNA gene sequence phylogeny (Hugenholtz et al., 2016). Due to the integration of genomic data into polyphasic taxonomy, a huge reclassification of bacteria has occurred in recent years (Gupta et al., 2018; Breider et

al., 2014; Orata et al., 2018). The genus *Rhodobacter* is taxonomically well studied, and some members are model organisms for biophysical and genetic engineering studies. However, this genus is comprised of a heterogeneous group of members. 16S rRNA gene-based phylogeny of the genus *Rhodobacter* indicates a motley assemblage of anoxygenic phototrophic bacteria (genus *Rhodobacter*) with interspersing members of other genera (chemotrophs) making the genus polyphyletic. The chemo-taxonomical differences among the *Rhodobacter* spp. was commented by Hiraishi and Ueda (1994) twenty five years back. However, due to a lack of strong evidence, reclassification of this genus has not yet been done. Therefore, to address this taxonomic discrepancy we propose reclassifications within the genus *Rhodobacter.*

4.2.1 Reclassification of *Gemmobacter changlensis* into *Cereibacter changlensis*

Rhodobacter changlensis (Anil Kumar et al., 2007) was reclassified as *Catellibacterium changlense* based on 16S rRNA gene sequence phylogeny and chemotaxonomic characters (Zheng et al., 2011). However, all the members of genus *Catellibacterium* were transferred into the genus *Gemmobacter* based on the phylogenetic, physiological, biochemical, fatty acid profiles, polar lipid profiles (Chen et al., 2013b). But, presence of an unidentified glycolipid, phototrophic growth, BChl *a*, internal membrane system, light harvesting complexes, absence of $C_{18:0}$3-OH (Table 3.6) and formation of separate clade in the 16S rRNA gene based phylogenetic tree necessitated separation of *G. changlensis* JA139^T and its related strains from the other *Gemmobacter* members. To accommodate these members a novel genus with the name *Cereibacter* gen. nov. is proposed.

4.2.1.1 Description of *Cereibacter* gen. nov.

Cereibacter [Ce.re.i.bac′ter. L. adj. *cereus* waxen, wax-′coloured; n. *bacter* a rod; N.L. masc. n. *Cereibacter* wax-coloured (brownish-yellow) rod].

Encompasses oval to rod shaped, non-motile, psychrotolerant, phototrophic bacteria which multiply by binary fission. Cells are Gram-stain negative. Internal photosynthetic membranes are present as vesicles. Photosynthetic pigments are, bacteriochlorophyll *a* and carotenoids of spheroidene series. Chemotrophic growth is possible. Growth factors are required for growth. Ubiquinones with 10 isoprene units are present. Major cellular fatty acid is $C_{18:1}\omega7c/C_{18:1}\omega6c$ with minor amounts of $C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega7c/C_{16:1}\omega6c$ and hydroxyl acid of $C_{10:0}$3OH are present. The G+C content of genomic DNA is 69.2–69.4 mol% (HPLC). Delineation of the genus is determined primarily by the phylogenetic information from 16S rRNA gene sequences, phototrophic growth, chemo-taxonomic and phenotypic traits. The type species is *Cereibacter changlensis.*

4.2.1.1.1 Description of *Cereibacter changlensis* comb. nov.

Basonym: *Gemmobacter changlensis* (Anil Kumar et al. 2007) Chen et al. 2013b

The description of the species as given by Anil Kumar et al, (2007), Zheng et al, (2011) and Chen et al. (2013b) is unchanged.

The type strain is JA139^T (= DSM18774^T = CCUG 53722^T = JCM 14338^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain AM399030 and that of the genome sequence is PZKG00000000.

4.2.2 Reclassification of *Rhodobacter* spp. into three new genera based on genome data

The use of genomics in taxonomic studies improves the credibility, reliability and reproducibility of the data, serves as an informative tool in establishing phylogenetic relationships among prokaryotes and helps in eliminating unreliable methods and subjective difficult-to-replicate data (Chun and Rainey, 2014; Chun et al., 2018; Pérez-Cataluña et al., 2018). To resolve the taxonomic conflict which have arisen due to heterogeneity among the *Rhodobacter* spp., genome based analysis was carried out. The genome size of clade I members is relatively larger than that of the remaining clades, with the exception of *Rba. ovatus* (Fig. 3.18; Table 3.8). The clade I members have higher G+C content (68.2-69.1 mol%) when compared with clade II (61.0-66.6 mol%), clade III (66.4-66.5 mol%), clade IV (65.0 mol%) and clade V (68.2 mol%). According to Nouioui et al., (2018) genome size variation can be a genus specific taxonomic marker, indicating that the clade I members might be belong to a distinct genus from *Rhodobacter sensu stricto* (Clade II). This is also in agreement with the separation of *Rba. capsulatus* strains from *Rba. sphaeroides* in the phylogenomic analysis of Simon et al. (2017).

Taxonomy of bacteria based on core- and pan-genome analysis is a powerful extension of the polyphasic approach (Inglin et al., 2018). Pan-genome analysis of the genus *Rhodobacter* revealed that the members have only 1239 core genes (Table 3.9). An increased number of core genes was observed when the clade wise pan genome analysis performed, which indicated the inter cluster genomes were divergent (Table 3.9, Fig. 3.19, Fig. 3.20, Fig. 3.21).

Digital DDH values (Table 3.10) among *Rhodobacter* members support the current species delineations, except between *Rba. sphaeroides* 2.4.1^{T} and *Rba.*

megalophilus JA194^T which exceeds the cut off of 70% *d*DDH value for prokaryotic species delineation (Wayne et al., 1984). This suggests that *Rba. megalophilus* JA194^T can be a sub-species of *Rba. sphaeroides* ATH 2.4.1^T. With the advancement in next generation sequencing methods, ANI is increasingly being used to delineate closely related species (Kim et al., 2014). Bacterial strains having less than 95-96% of ANI are considered as distinct species (Rodriguez and Konstantinidis, 2014; Goris et al., 2007; Richter and Rosselló-Móra, 2009). In agreement with the *d*DDH data, OrthoANI values also confirm that all species of *Rhodobacter* are well described at species level, exception for *Rba. megalophilus* which gave an ANI score of 97.96% with *Rba. sphaeroides* ATH 2.4.1^T (Table 3.10), confirming these two strains belong to the same species.

Qin et al. (2014) proposed that if the POCP values between two species were less than 50% then they belong to two different genera. However, this threshold does not appear to apply among members of *Rhodobacter* (Table 3.11), suggesting that they all belong to a single genus. POCP cut off values are ineffective in delineating genera of the family 'Rhodobacteraceae' (Wirth and Whitman 2018), *Methylococcaceae* (Orata et al., 2018) and *Neisseriaceae* (Li et al., 2017) suggesting a need to establish appropriate POCP cut-off values for different families.

Percentage of AAI below 60% between the compared genomes of species was also proposed to delineate genera (Rodriguez and Konstantinidis, 2014). However, the AAI values between members of related but different genera can vary between 60-80% (Luo et al., 2014; Orata et al., 2018). The similarity index of AAI between *Rhodobacter* spp. (Table 3.11) strengthen the proposal for reclassifying them, while the phylogenetically related genera (chemotrophs) in the family 'Rhodobacteraceae' are well described and require no further reclassification. The phylogenomic tree

topology using genomic DNA sequences (UBCG; Fig. 3.17) or protein sequences (Fig. 3.27, Fig. 3.28) was in congruence with the 16S rRNA gene-based phylogeny (Fig. 3.16). The phylogenetic tree based on the proteins (Fig. 3.24, Fig. 3.24, Fig. 3.26) involved in photosynthesis also showed the polyphyletic assemblage of the *Rhodobacter* spp. which indicates the need of reclassification of some members of the genus *Rhodobacter*.

The conflict observed in the current classification of the genus *Rhodobacter* is visible in single gene based trees, either in the 16S rRNA gene (Fig. 3.16) or *rpoB* gene based tree (Fig. 3.8), which is strengthened by better resolved whole genome phylogenies. The results of phylogenomic analysis emphasise the need for reclassification of the taxonomically complex genus *Rhodobacter*. Housekeeping genes (such as *rpoB*) can be used to determine the phylogenetic relationships of species when the 16S rRNA gene sequence based phylogenetic tree is unable to resolve the phylogenetic position of taxa, based on the fact that the house keeping genes are less conversed and evolve faster than the 16S rRNA gene (Martens et al., 2008). Based on the 16S rRNA gene and *rpoB* gene phylogenetic trees we believe that genome sequences for the remaining four members (*Rba. alkalitolerans*, *Rba. azollae*, *Rba. lacus* and *Rba. sediminis*) are not essential to resolve the taxonomic positions of members of the genus *Rhodobacter*.

4.2.2.1 Phenotypic and chemotaxonomic characters between the *Rhodobacter* clades

The members of *Rba. sphaeroides* clade (clade I) were isolated from freshwater environments only (Gandham et al., 2018, Srinivas et al., 2008, Girija et al., 2010), whereas *Rba. capsulatus* clade (clade II) members have been isolated from freshwater (Suresh et al., 2017), estuarine (Venkata Ramana et al., 2009) and marine

habitats (Venkata Ramana et al., 2008). *Rba. blasticus*, which is assigned to clade III, and *Rba. veldkampii* (clade IV member), were isolated from freshwater habitats (Eckersley and Dow, 1980 and Hansen and Imhoff, 1985), while *Rba. vinaykumarii* was isolated from a marine habitat (Srinivas et al., 2007). However, the isolation source is not the distinguishing feature between the clades. The members of clade II are motile (Suresh et al., 2017), whereas some of the members of clade I do not exhibit motility, and members of clade III, IV and clade V are not motile (Eckersley and Dow, 1980, Hansen and Imhoff, 1985, Srinivas et al., 2007). Except for *Rba. blasticus* which multiplies by sessile budding and has a lamellar ICM, the remaining species of *Rhodobacter* multiply by binary fission and have a vesicular architecture of their intracytoplasmic membranes (Table 3.12). Only the clade V member (*Rba. vinaykumarii* JA123^T) has a requirement of NaCl for optimum growth and cannot assimilate sulfate (Srinivas et al., 2007). Only one member (*Rhodobacter azotoformans*) of clade I has denitrification ability (Hiraishi et al., 1996).

All the members of clade I (*Rba. sphaeroides*, *Rba. johrii*, *Rba. megalophilus*, *Rba. azotoformans*, *Rba. ovatus* and *Rba. alkalitolerans*) have glycolipid and DPG (Fig. 3.9, Fig. 3.29). Polar lipid analysis has been a central tool in chemotaxonomy and the presence/absence of glycolipid was considered as one of the genus differentiating characters in the reclassification of some species of the genus *Rhodobium* into a new genus, *Afifella* (Urdiain et al., 2008). Members of clade II, III, IV and V do not have DPG or glycolipid (Fig. 3.5, Fig. 3.29). Except for some of members of clade II, all members of the new genera proposed below have spheroidene as their major carotenoid (Suresh et al., 2017, Gandham et al., 2018, Girija et al., 2010). The chemotaxonomic comparison between the genus *Rhodobacter*

sensu stricto, the proposed novel genera and closely related genera is given in the Table 3.12.

4.2.2.2 Justification for separating *Rhodobacter* spp. into new genera

Phenotypic and phylogenomic features support the separation of genus *Rhodobacter* into four different genera. Emended descriptions and reclassifications are given for these taxa. Apart from the tree topologies, with interspersing chemotrophic members, the branch lengths in the whole-genome and 16S rRNA trees indicated that the *Rhodobacter* species are too divergent to be placed into the same genus. Since its description, five out of 21 originally proposed *Rhodobacter* species have been reclassified into different genera and it has been emphasized that *Rhodobacter* is still heterogeneous and should be subdivided based on additional molecular taxonomic data (Hiraishi and Ueda, 1994; Lee et al., 2005; Helsel et al., 2007; Suresh et al., 2015).

16S rRNA gene and *rpoB* gene (Gandham et al., 2018) based phylogenetic trees shows that *Rba. alkalitolerans* JA916^{T} together with *Rba. johrii*, *Rba. megalophilus*, *Rba. sphaeroides*, *Rba. azotoformans* and *R. ovatus* fall outside of the *Rba. capsulatus* clade and belong in what we define as the *Rba*. *sphaeroides* clade. We propose that the members of the *Rba*. *sphaeroides* clade be classified into a new genus for which we propose the name *Luteovulum* gen. nov. Moreover, as *Rba. sphaeroides* 2.4.1^{T} and *Rba*. *megalophilus* JA194^{T} have high genomic indices (OrthoANI and *d*DDH) we conclude that they are not two different species. Based on phenotypic differences (Arunasri et al., 2008), we propose *Rba. megalophilus* as a sub-species of *Rba. sphaeroides*.

Rba. viridis, *Rba. azollae*, *Rba. sediminis*, *Rba. capsulatus*, *Rba. aestuarii*, *Rba. lacus* and *Rba. maris* form a distinct clade in both the single gene trees (*rpoB*

gene and 16S rRNA gene). This clade accommodates the type species of the genus *Rhodobacter*, *Rba. capsulatus* and members of this clade are proposed to be included in the genus *Rhodobacter sensu stricto*. *Rba. veldkampii* and *Rba. vinaykumarii* formed two different clusters with interspersing chemotrophs. However, in the genome-based trees (UBCG based, proteome based) they clustered together. Genome based phylogenomic trees have better resolution than single gene-based trees, so for the time being we are not separating these two into two different genera, which can be done in future based on multiple species/strains.

For the *Rba. veldkampii* clade accommodating *Rba. vinaykumarii* and *Rba. veldkampii*, we propose the new genus *Phaeovulum* gen. nov. To accommodate *Rba. blasticus*, we propose the new genus *Fuscovulum* gen. nov. The classification of *Rba. blasticus* itself controversial when it was transferred from the genus *Rhodopseudomonas* to *Rba. blasticus*, despite having lamellar ICM structures in contrast to all other members of genus *Rhodobacter*, which have vesicular ICM structures. It was proposed that ICM systems may be of questionable value as genus specific characters, because they are not coincident with the 16S rRNA gene phylogenetic relationships (Kawasaki et al., 1993). Later Hiraishi and Ueda (1994) suggested separating *Rba. veldkampii* and *Rba. blasticus* from the genus *Rhodobacter* into a novel genus based on the ICM architecture, mode of cell division of *Rba. blasticus*, and final oxidation product of sulfide in the case of *Rba. veldkampii*. Hiraishi and Ueda (1994) considered that mode of cell division and ICM types are genus specific characters. Here we propose the reclassification of each of these species into separate genera which, after two and half decades, helps to resolve the taxonomic conflict within the genus *Rhodobacter*, based on the genomic and chemotaxonomic information.

4.2.2.3 Emended description of the genus *Rhodobacter* (Imhoff et al. 1984) Srinivas et al. 2007

Members can be isolated from freshwater ponds, estuarine and marine environments. Oval-to-rod shaped cells have vesicular ICM architecture. Cells are mostly motile with a single polar flagellum and multiply by binary fission. Catalase and oxidase positive. Primarily phototrophic and contain BChl *a* and carotenoids of the spheroidene series. Aerobic growth occurs in most species. Mesophilic. Phototrophic growth occurs on a range of organic substrates. The growth factors biotin, niacin and thiamine, alone or in combination, are required for growth to occur. NaCl requirement is not obligatory, can tolerate up to 2-3%. $C_{18:1}\omega7c/C_{18:1}\omega6C$, $C_{18:0}$, $C_{16:0}$ and $C_{16:1}$ $\omega7C/C_{16:1}\omega6c$ are the major fatty acids. Most species have $C_{10:0}3OH$ and $C_{18:0}3OH$ as fatty acid alcohols. Phosphatidylethanolamine, phosphatidylglycerol and phosphatidylcholine are the major polar lipids. Glycolipid and diphosphatidylglycerol are absent. Hopanoids are not produced. Q-10 is the major quinone.

The type species is *Rhodobacter capsulatus* (Molisch 1907) Imhoff et al. 1984.

4.2.2.4 Taxonomic note on *Rhodobacter megalophilus* Arunasri et al., 2008

Based on the OrthoANI and *d*DDH analysis, *Rba. megalophilus* is not a distinct species as the differences between *Rba. sphaeroides* and *Rba. megalophilus* represent intra-species divergence. It should be noted that *Rba. sphaeroides* and *Rba. megalophilus* differ with regard to growth at 5 ˚C, absence of flagellar motility, cell suspension colour, vitamins required for growth, ability to utilise sulfide or hydrogen as electron donors and ability to utilise citrate as carbon source (Arunasri et al., 2008). Based on chemotaxonomic data we propose that *Rba. megalophilus* be reclassified as a sub-species of *Rba. sphaeroides*.

4.2.2.5 Description of *Luteovulum* gen. nov.

Luteovulum (Lu.te.o'vu.lum. L. adj. *luteus* yellow; N.L. dim. neut. n. *ovulum* (from L. n. *ovum*, an egg) a small egg; N.L. neut. n. *Luteovulum* small yellow egg).

Members can be isolated from freshwater ponds, paddy soils, wastewater treatment plants, alkaline ponds and lake sediments. Gram-stain negative, oval-to-rod shaped cells and have vesicular ICM architecture. Cells are mostly motile with a single polar flagellum and multiply by binary fission. Catalase and oxidase positive. Primarily phototrophic and contain BChl *a* and carotenoids of the spheroidene series. Facultative aerobes and mesophilic. Phototrophic growth occurs on a number of organic substrates. The growth factors biotin, niacin and thiamine, alone or in combination, are required for growth. NaCl requirement is not obligatory, can tolerate up to 2-3%. $C_{18:1}\omega7c/C_{18:1}$ ω6C, $C_{18:0}$, $C_{16:0}$, $C_{10:0}$ 3OH, $C_{16:1}$ ω7C/$C_{16:1}$ ω6c, $C_{18;1}\omega7c11methyl$ are the major fatty acids. Phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and an unidentified glycolipid are the major polar lipids. Hopanoids are not produced. Q10 is the major quinone. Delineation of the genus is based on 16S rRNA and *rpoB* gene-based phylogeny, phylogenomics, genome comparison and chemotaxonomic differences.

The type species is *Luteovulum sphaeroides* ATH 2.4.1

4.2.2.5.1 Description of *Luteovulum sphaeroides* comb. nov.

Luteovulum sphaeroides (N.L. *sphaera*, sphere, globe; L. suff. *-oides* (from Gr. suff. *-eides*, from Gr. n. *eidos*, that which is seen, form, shape, figure), ressembling, similar; N.L. masc. adj. *sphaeroides*, spherical).

Basonym: *Rhodobacter sphaeroides* Imhoff et al. 1984

The description of *Luteovulum sphaeroides* is identical to that of *Rba. sphaeroides* (Imhoff et al., 1984; Imhoff, 2005) except for the following modifications. $C_{17:0}$ is present in minor quantities. An unidentified aminolipid, an unidentified phospholipid and few unidentified lipids are additional polar lipids, growth can occur at 5 ˚C, some strains are non-motile. Type strain is available from the DSMZ (DSM 158^T) and LMG (LMG 2827^T).

4.2.2.5.1.1 Description of *Luteovulum sphaeroides* subsp. *sphaeroides* subsp. nov.

The description of *Luteovulum sphaeroides* is identical to that of *Rba. sphaeroides* (Imhoff et al., 1984; Imhoff, 2005) except for the following modifications. $C_{17:0}$ is present in minor quantities. An unidentified aminolipid, an unidentified phospholipid and two unidentified lipids are additional polar lipids. Type strain (*Luteovulum sphaeroides* ATH 2.4.1) is available from the DSMZ (DSM 158^T) and LMG (LMG 2827^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is X53853 and CP030271, CP030272, CP030273, CP030274, CP030275 and CP030276 are the genome sequence accession numbers of the type strain.

4.2.2.5.1.2 Description of *Luteovulum sphaeroides* subsp. *megalophilum* subsp. nov.

Luteovulum megalophilum (me.ga.lo'phi.lum. Gr. adj. *megas*, wide; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*) friend, loving; N.L. neut. adj. *megalophilum*, wide (temperature)-loving).

The description of *Luteovulum megalophilum* is identical to that of *Rba. megalophilus* (Arunasri et al., 2008) except for the following modifications. 3-Hydroxy $C_{10:0}$ and $C_{12:0}$ fatty acids are present. The DNA G+C content of the type strain calculated from genome sequence is 68.8 mol%. The type strain (*Luteovulum megalophilum* JA194^T)

is available from JCM (JCM 14598^T) and KCTC (KCTC 5602^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain AM421024 and that of the genome sequence is FZOV00000000.

4.2.2.5.2 Description of *Luteovulum johrii* comb. nov.

Luteovulum johrii (joh′ri.i. N.L. masc. gen. n. *johrii* of B. N. Johri, an eminent and well-known Indian microbiologist).

Basonym: *Rhodobacter johrii* Girija et al. 2010

The description of *Luteovulum johrii* is identical to that of *Rba. johrii* (Girija et al., 2010). The type strain (*Luteovulum johrii* JA192^T) is available from the JCM (JCM 14543^T) and DSMZ (DSM 18678^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is and that of the genome sequence is MABH00000000.

4.2.2.5.3 Description of *Luteovulum ovatum* comb. nov.

Luteovulum ovatum (o.va'tum. L. neut. adj. *ovatum*, egg-shaped, ovate)

Basonym: *Rhodobacter ovatus* Srinivas et al. 2008

The description of *Luteovulum ovatum* is identical to that of *Rba. ovatus* (Srinivas et al., 2008). Type strain (*Luteovulum ovatum* JA234^T) is available at JCM (JCM 14779^T) and CCUG (CCUG 55049^T). AM690348 is the 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number and OAOQ00000000 is the genome sequence accession number.

4.2.2.5.4 Description of *Luteovulum azotoformans* comb. nov.

Luteovulum azotoformans (a.zt.to.for'mans. N.L. n. *azotum* [from French n. azote (from Gr. prep. *a*, not; Gr. n. *zôê*, life; N. Gr. n. *azôê*, not sustaining life)], nitrogen; N.L. pref. *azo-*, pertaining to nitrogen; L. part. adj. *formans*, forming; N.L. part. adj. *azotoformans*, nitrogen forming).

Basonym: *Rhodobacter azotoformans* Hiraishi et al. 1997

The description of *Luteovulum azotoformans* is identical to that of *Rba. azotofarmans* (Hiraishi et al., 1997). The type strain (*Luteovulum azotoformans* KA25^T) is available from JCM (JCM 9340^T) and NBRC (NBRC 16436^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is D70846 and that of the genome sequence is QAOT00000000.

4.2.2.5.5 Description of *Luteovulum alkalitolerans* comb. nov.

Luteovulum alkalitolerans (al.ka.li.to'le.rans. N.L. n. *alkali*, alkali; L. part. adj. *tolerans*, tolerating; N.L. part. adj. *alkalitolerans*, alkali-tolerating).

Basonym: *Rhodobacter alkalitolerans* Gandham et al. 2019

The description of *Luteovulum alkalitolerans* is identical to that of *Rba. alkalitolerans* (Gandham et al., 2019). The type strain (*Luteovulum alkalitolerans* JA916^T) is available from the KCTC (KCTC 15473^T) and LMG (LMG 28749^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is LN810645.

4.2.2.6 Description of *Fuscovulum* gen. nov.

Fuscovulum (Fusc.o'vu.lum. L. masc. adj. *fuscus*, tawny N.L. dim. neut. n. *ovulum* (from L. n. *ovum*, an egg), a small egg; N.L. neut. n. *Fuscovulum* small tawny egg).

Members can be isolated from freshwater habitats. Gram-stain negative, rod-to-oval shaped cells have lamellar ICM architecture. Cells are mostly non motile and multiply by sessile budding. Catalase and oxidase positive. Primarily phototrophic and contain BChl *a* and carotenoids of the spheroidene series. Growth occurs under aerobic, anaerobic and mesophilic conditions. Phototrophic growth occurs on a number of organic substrates. Nicotinic acid and thiamine are required for growth. NaCl is not required for growth. Q-10 is the major quinone. The G+C mol% of the type strain of the type species is 66.5%. Delineation of the genus is based on 16S rRNA and *rpoB* gene-based phylogeny, phylogenomics, genome comparison and chemotaxonomic differences.

The type species is *Fuscovulum blasticum.*

4.2.2.6.1 Description of *Fuscovulum blasticum* comb. nov.

Fuscovulum blasticum (Gr. adj. *blastikos -ê -on*, budding, sprouting; N.L. masc. adj. *blasticum*, budding, apt to bud).

Basonym: *Rhodobacter blasticus* corrig. (Eckersley and Dow 1980) Kawasaki et al. 1994.

The description of *Fuscovulum blasticum* is identical to that of *Rhodopseudomonas blastica* (Eckersley and Dow, 1980) and *Rhodobacter blasticus* (Imhoff et al., 1984; Imhoff, 2005). The type species is available from the ATCC (ATCC 33485^T) and NBRC (NBRC 16437^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is DQ342322 and that of the genome sequence is PZKE00000000.

4.2.2.7 Description of *Phaeovulum* gen. nov.

Phaeovulum (Phae.o'vu.lum. N.L. neut. adj. *phaeum* (from Gr. neut. adj. phaion), brown;

N.L. dim. neut. n. *ovulum* (from L. n. *ovum*, an egg) a small egg; N.L. neut.

n. *Phaeovulum* small brown egg)

Members can be isolated from marine habitats including tidal waters, fresh water. Gram-stain negative, rod to oval shaped cells have vesicular ICM architecture. Cells are mostly non-motile and multiply by binary fission. Catalase and oxidase positive. Primarily phototrophic and contain BChl *a* and carotenoids of the spheroidene series. Growth occurs under anaerobic and mesophilic conditions. Phototrophic growth occurs on a number of organic substrates. Biotin, *para*-aminobenzoate and thiamine are required for growth either singly or in combination. Some strains do not contain phosphatidylcholine. Some require NaCl for growth and can tolerate up to 4%. $C_{18:1}$ ω7c/$C_{18:1}$ ω6C, $C_{18:0}$, $C_{16:0}$, $C_{10:0}$ 3OH, $C_{16:1}$ ω7C/$C_{16:1}$ ω6c, $C_{18;1}$ ω7c11methyl are the major fatty acids. Q-10 is the major quinone. G+C mol% is 65-68.25. Delineation of the genus is based on 16S rRNA and *rpoB* gene based phylogeny, phylogenomics, genome comparison and chemotaxonomic differences.

The type species is *Phaeovulum veldkampii*.

4.2.2.7.1 Description of *Phaeovulum veldkampii* comb. nov.

Phaeovulum veldkampii (veld. kamp'i.i. N. L. gen. masc. n. *veldkampii* of Veldkamp; named for Hans Veldkamp, a Dutch microbiologist).

Basonym: *Rhodobacter veldkampii* Hansen and Imhoff, 1985.

The description of *Phaeovulum veldkampii* is identical to that of *Rhodobacter veldkampii* (Hansen and Imhoff, 1985; Imhoff, 2005). The type strain is available from the ATCC (ATCC 35703^T) and DSMZ (DSM 11550^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number is D16421 and that of the genome sequence is PZKF00000000.

4.2.2.7.2 Description of *Phaeovulum vinaykumarii* comb. nov.

Phaeovulum vinaykumarii (vi′nay.ku.ma′ri.i. N.L. masc. gen. n. *vinaykumarii* of Vinaykumar, named after the late Dr M. Vinaykumar, an Indian microbiologist and research supervisor of Ch.V.R. and Ch.S, who initiated work on anoxygenic phototrophic bacteria in India).

Basonym: *Rhodobacter vinaykumarii* Srinivas et al., 2007.

The description of *Phaeovulum vinaykumarii* is identical to that of *Rhodobacter vinaykumarii* (Srinivas et al., 2007). The type strain is available from the JCM (JCM 14544^T) and DSMZ (DSM 18714^T). The 16S rRNA gene sequence GenBank/EMBL/DDBJ accession number of the type strain is AM408117 and that of the genome sequence is OBMN00000000.

4.3 Taxonomic note on recently published *Rhodobacter* spp. [*Rhodobacter thermarum* (Khan et al., 2019), *Rhodobacter flagellatus* (Xian et al., 2019), *Rhodobacter sediminicola* (Suresh et al., 2019a)]

Recently, three new species i.e., *Rhodobacter sediminicola* (Suresh et al., 2019a), *Rhodobacter thermarum* (Khan et al., 2019), *Rhodobacter flagellatus* (Xian et al., 2019) were added to the genus *Rhodobacter*. In 16S rRNA gene sequence based (Fig. 4.1), genome (UBCG) based phylogenetic trees (Fig. 4.2), *Rba. sedminicola* was cladding with *Rba. sphaeroides* clade [reclassified as *Luteovulum* gen. nov. (Suresh et al., 2019b)], suggesting that the *Rba. sedminicola* might be belonging to genus *Luteovulum* gen. nov. More than 80% AAI score (Table 4.1) among the *Rba. sphaeroides* clade members including *Rba. sediminicola* confirmed the necessity to transfer into the genus *Luteovulum* gen. nov.

Rhodobacter thermarum, *Rhodobacter flagellatus* were forming sub-clade with *Rba. blasticus* (named as *Fuscovulum blasticus*) in both 16S rRNA gene

sequence (Fig. 4.1) and genome based phylogenetic trees (Fig. 4.2). The phenotypic differences like isolation source, ability to grow at high temperatures, binary fission mode of cell division, vesicular/lamellar ICM structures are separating *Rhodobacter thermarum* and *Rhodobacter flagellatus* from *Fuscovulum blasticus*. Less the 80% AAI score (Table 4.1) was confirming that the *Rhodobacter thermarum* and *Rhodobacter flagellatus* were not belonging to the genus *Fuscovulum* and must be reclassified into a new genus in future. The AAI score between the *Rhodobacter thermarum* and *Rhodobacter flagellatus* is more than 80% (Table 4.1) and similar chemotaxonomic characters indicated that they both belong to same genus.

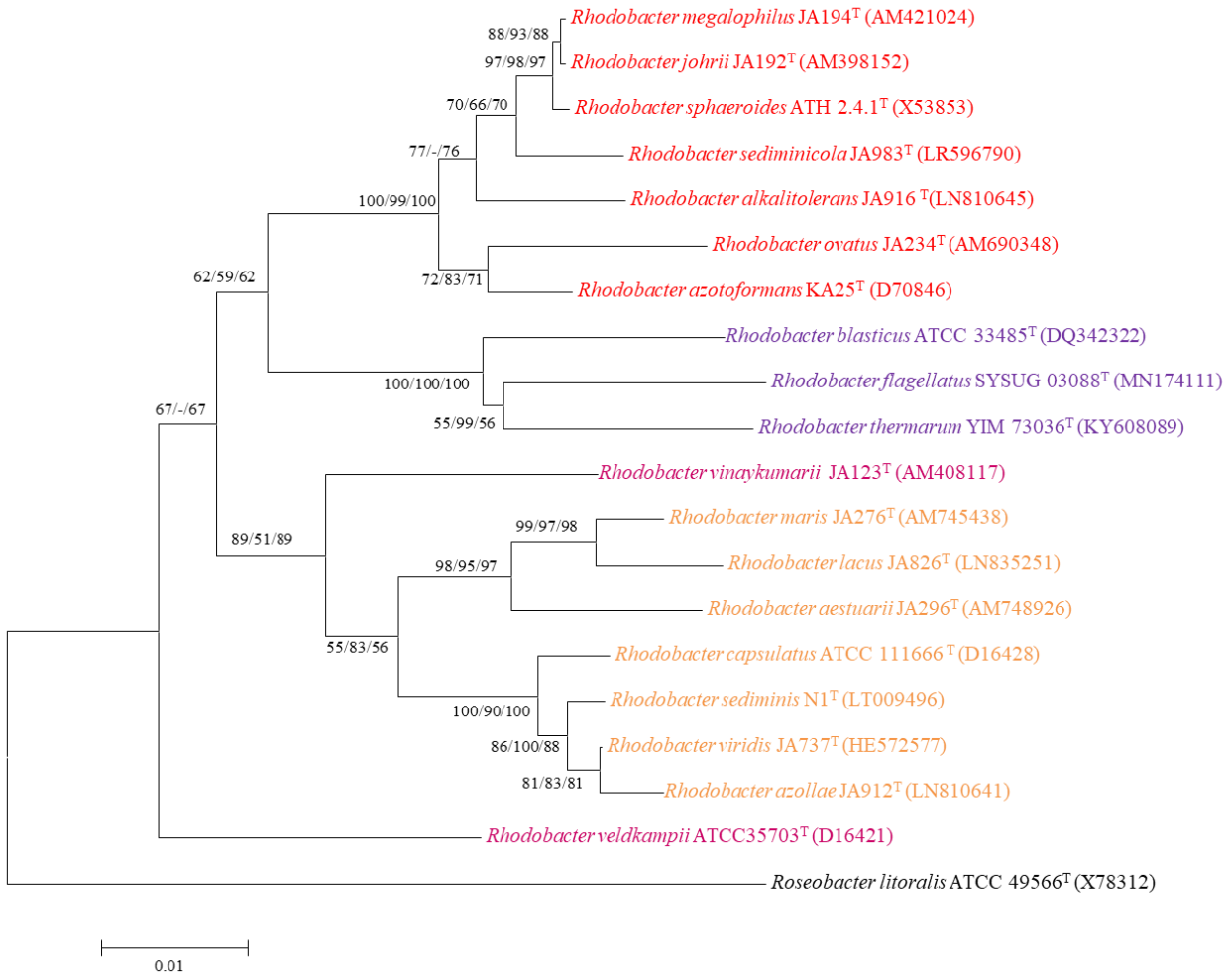

Fig. 4.1 Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship of newly described *Rhodobacter* spp. with the members of genus *Rhodobacter*

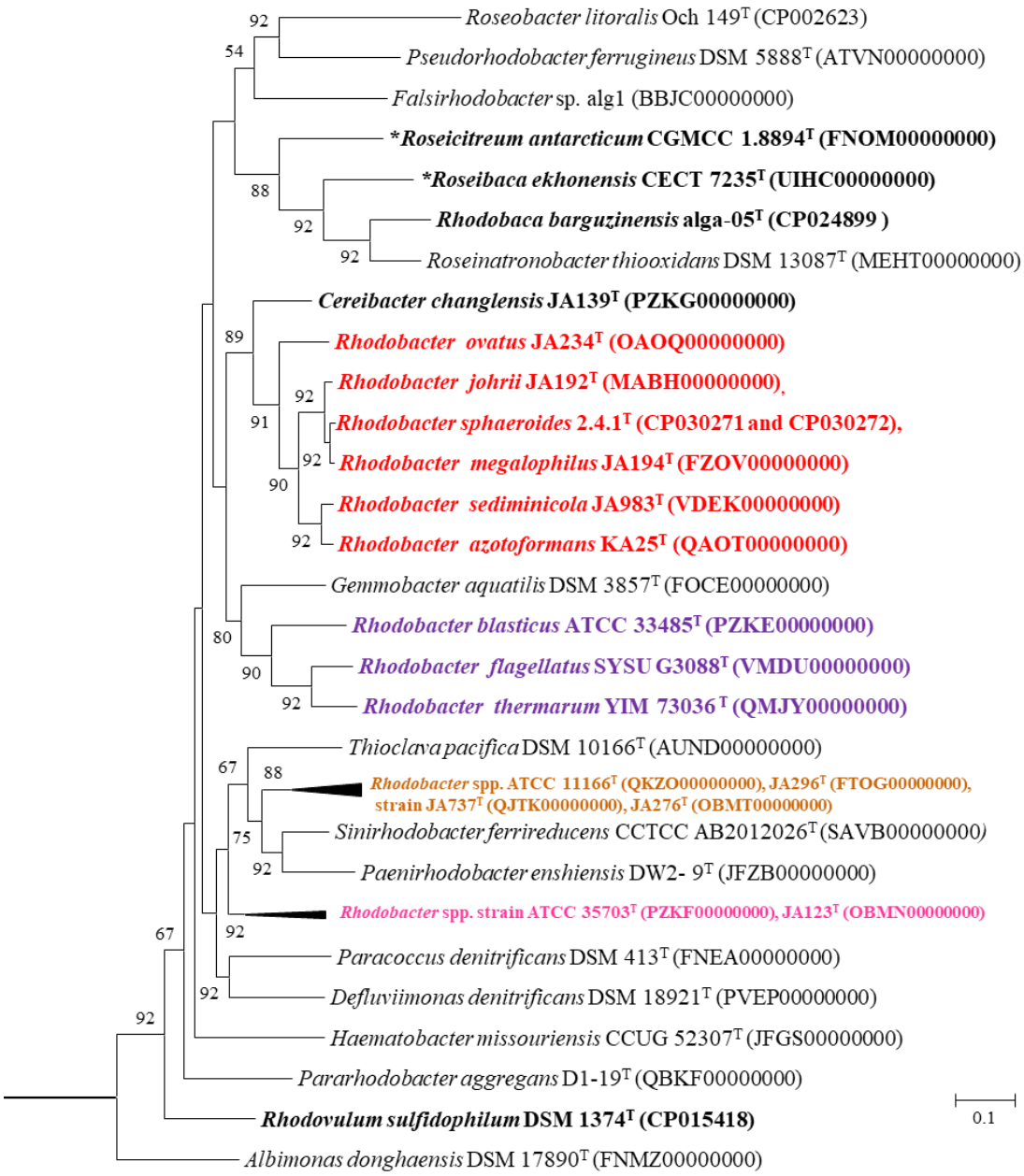

Fig. 4.2 Phylogenomic tree constructed using the Ninety-two bacterial core gene sequences of newly described *Rhodobacter* spp. and related members of family 'Rhodobacteraceae'. Bootstrap percentages refer to Neighbor Joining analysis. Phototrophic bacteria are indicated by bold letters and aerobic anoxygenic phototrophic bacteria with bold letters and star.

Table 4.1 AAI values between newly described *Rhodobacter* members and *Rba. sphaeroides, Rba. blasticus* clade members

	1	2	3	4	5	6	7	8	9
1									
2	96.32								
3	98.56	96.39							
4	86.42	86.47	86.09						
5	80.94	81.05	80.37	81.08					
6	86.38	86.50	86.69	96.12	82.00				
7	67.25	67	66.92	66.75	68	67.05			
8	66.23	66.39	6596	66.34	68.03	67.32	71.13		
9	66.68	66.61	65.98	66.33	68.05	67.48	72.16	86.22	

Taxa; 1- *Rhodobacter sphaeroides* 2.4.1^T; 2- *Rhodobacter johrii* JA192^T; 3- *Rhodobacter megalophilus* JA194^T; 4- *Rhodobacter azotoformans* KA25^T, 5- *Rhodobacter ovatus* JA234^T; 6- *Rhodobacter sediminicola* JA983^T; 7- *Rhodobacter blasticus* DSM 213^T, 8- *Rhodobacter thermarum* YIM 73036^T, 9- *Rhodobacter flagellatus* SYSU G03088^T

4.3.1 Description of *Luteovulum sediminicola* comb. nov.

Luteovulum sediminicola (se.di.mi.ni'co.la. L. neut. N. *sedimen* sediment; L. suff. –*cola* (from L. masc. or fem. N. *incola*) inhabitant, dweller; N.L. masc. n. *sediminicola* dweller of sediments)

Basonym: *Rhodobacter sediminicola* Suresh et al. 2019a

The description of *Luteovulum sediminicola* is identical to that of *Rba. sediminicola* (Suresh et al., 2019a). The type strain (*Luteovulum sediminicola* JA983^T) is available from the KCTC (KCTC 15782^T) and NBRC (NBRC 113843^T). The GenBank accession number for the 16S rRNA gene sequence of *Luteovulum sediminicola* JA983^T is LR596790 and the draft genome has been deposited in GenBank under the accession number VDEK000000000.

4.4 Taxonomic note on *Cereibacter changlensis* (Suresh et al., 2015)

In the month of January 2020, Hameed et al. (2020) reclassified *Cereibacter changlensis* again back to 'Gemmobacter changlensis' based on the 16S rRNA gene sequence phylogenetic and phenotypic characters, but they missed use of genome data in this reclassification. However, while studying the *Rhodobacter* taxonomy, we have found that less than 80% AAI (Table 4.1) score between the *Gemmobacter aquatilis* (type species) and *Cereibacter changlensis*, cladding pattern in genome based trees (Table 4.1) are in-favour of *Cereibacter changlensis* proposal. Hence, *Cereibacter changlensis* nomenclature will retain as it is.

Summary

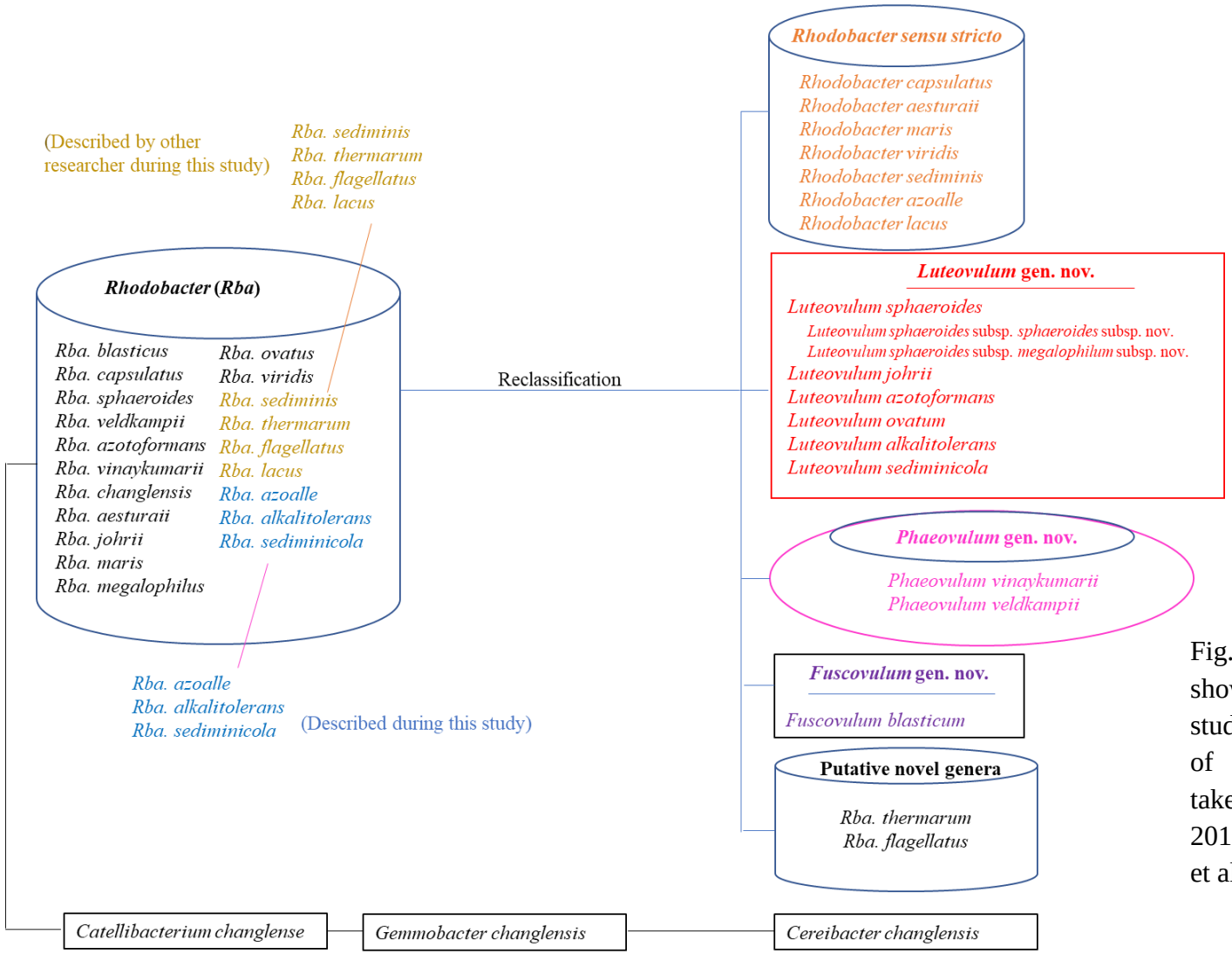

Fig. 5.1 Graphical representation showing the summary of the present study and present taxonomic status of the genus *Rhodobacter*. Data taken from Suresh et al. (2015; 2019a,b); Xian et al. (2019); Khan et al. (2019).

Major findings

6. Major Findings

- Three strains were described as new species of genus *Rhodobacter*; *Rba. azollae* sp. nov., *Rba. alkalitolerans* sp. nov., and *Rba. sediminicola* sp. nov.
- In addition to the major objectives of the thesis, a strain of *Rhodomicrobium* which was isolated from Umiam lake during the study was also described as a new species as *Rhodomicrobium lacus* sp. nov. JA980^{T} (Suresh et al., 2020).
- The following reclassifications were done in the genus *Rhodobacter*.
  - *Rhodobacter megalophilus* as a sub-species of *Rhodobacter sphaeroides*.
  - *Gemmobacter changlensis* (*Rhodobacter changlensis*) to *Cereibacter changlensis*.
  - Based on taxogenomic and polyphasic analysis, three new genera are proposed, *Luteovulum* gen. nov., *Fuscovulum* gen. nov., *Phaeovulum* gen. nov. along with emended description of *Rhodobacter sensu stricto*.

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Publications

8. Publications

1. **Suresh, G.,** Dhanesh, Kumar., Krishnaiah, A., Sasikala, Ch., and Ramana, Ch.V. (2019). *Rhodobacter sediminicola* sp. nov., isolated from a fresh water pond of Gujarat. *Int. J. Syst. Evol. Microbiol.* https://doi.org/10.1099/ijsem.0.003913.
2. **Suresh, G.,** Lodha, T.D., Indu, B., Sasikala, C., and Ramana, C.V. (2019). Taxogenomics Resolves Conflict in the Genus *Rhodobacter*: A Two and Half Decades Pending Thought to Reclassify the Genus *Rhodobacter*. *Front. Microbiol.* 10, 2480.
3. **Suresh, G.,** Sailaja, B., Ashif, A., Dave, BP., Sasikala, Ch and Ramana, Ch.V (2017). Description of *Rhodobacter azollae* sp. nov. and *Rhodobacter lacus* sp. nov. *Int. J. Syst. Evol. Microbiol.* 67, 3289–3295.
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Conferences and Seminars

13. **Suresh, G**., Ramana, Ch.V., and Sasikala, Ch. (2018). *Luteovulum sediminicola* gen. nov. sp. nov., and reclassification of a few *Rhodobacter* spp. into three new genera". Poster presentation in 59th Annual conference of Association of Microbiologists of India during 9th-12th December, 2018 in Hyderabad, India.
14. **Suresh, G**., and Ramana Ch.V. (2017). Comparative analysis of photosynthetic gene cluster in the genus *Rhodobacter*. Poster presentation in 8th international conference on "Photosynthesis and Hydrogen Energy Reseach for Sustainability-2017" Oct 30th - Nov 03rd 2017, Hyderabad, India.
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MICROBIOLOGY SOCIETY

Rhodobacter sediminicola sp. nov., isolated from a fresh water pond

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Abstract

A phototrophic bacterium, designated as strain JA983^T, was isolated from a freshwater pond in Gujarat, India. The strain was yellowish brown, catalase- and oxidase-positive, rod-to-oval shaped, Gram-stain-negative and motile. Growth was observed at 20–35 °C. NaCl was not required for optimum growth and up to 5% was tolerated. Growth was observed at pH 6.0–8.0, with an optimum at pH 7.0. An unidentified glycolipid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, two unidentified aminolipids (AL1, AL2) and two unidentified lipids (L1 and L2) are the polar lipids of JA983^T. Q10 is the only quinone. $C_{18:1}\,\omega7c/C_{18:1}\,\omega6c$ is the major fatty acid. JA983^T showed highest 16S rRNA gene sequence similarity with the type strains of *Rhodobacter sphaeroides* (98.99%), *Rhodobacter megalophilus* (98.99%), *Rhodobacter johrii* (98.99%) and other members of the genus *Rhodobacter* with less than 98.7% similarity. In a 16S rRNA gene sequence-based phylogenetic tree, JA983^T formed a different sub-clade with its nearest phylogenetic members of genus *Rhodobacter*. Phenotypic, chemotaxonomic, phylogenetic and genomic [average nucleotide identity (ANI) and digital DNA–DNA hybridization (*d*DDH) differences indicated that JA983^T is significantly different from other species of the genus *Rhodobacter* and thus represents a novel species of the genus for which the name *Rhodobacter sediminicola* sp. nov. is proposed. The type strain is JA983^T (=KCTC 15782^T=NBRC 113843^T).

The genus *Rhodobacter* has 16 species with validly published names, at the time of writing, and members are primarily phototrophic, mesophilic organisms with no NaCl requirement for growth (except for *Rhodobacter vinaykumarii*) [1–5]. In our continuous search for and discovery of novel bacterial taxa from Gujarat, we have isolated some phototrophic bacteria, one of which was strain JA983^T. This study was performed to determine the phylogenetic position of strain JA983^T, isolated from a sediment sample.

Sediment from a fresh water pond having a salinity of 0.3% and pH of 6.5–7.0 was collected from Gujarat, India (GPS position: 21°60′N 72°30′E). The sample was kept for enrichment in a 40 ml transparent glass bottle in a medium described previously [3] for one week, at 30 °C in the light (2400 lux). Yellowish to brown colored growth appeared in the enrichment glass bottle, which was further purified by repeated streaking anaerobically and designated as strain JA983^T. The same medium was used for further purification and characterization (photoheterotrophic conditions). Pure culture was maintained on agar slants or as lyophilized cultures, preserved at 4 °C and also in 40% (v/v) glycerol, at −20 °C

Cell morphological studies were carried out on samples from cultures grown at 30 °C and at neutral pH. Cell division, shape, motility and size were observed under phase contrast or scanning electron microscope (BH-2; Olympus or XL3O, Philips). A transmission electron microscope was used for the detection of flagella and for the type of internal photosynthetic membranes. Uranyl acetate was used as a negative stain to determine the flagellar position and number. Cells were processed according to the protocol of Hanada *et al.* [6] for detection of internal membrane architecture. The Gram reaction was tested using a Gram staining kit (Himedia) according to the instructions given by the manufacturer. Growth at different temperatures (4, 8, 15, 20, 25, 30, 35 and 40 °C) was investigated at pH 7.0 and 0.05% w/v NaCl. Optimum pH for

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Keywords: *Rhodobacter sediminicola* sp. nov; freshwater pond.
Abbreviations: Bchl-*a*, Bacteriochlorophyll *a*; *d*DDH, Digital DNA-DNA Hybridization; HPLC, High-Pressure Liquid Chromatography; Ortho ANI, Orthologous Average Nucleotide Identity.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA and genome sequence of JA983^T are LR596790 and VDEK00000000, respectively.
Five supplementary figures and two supplementary tables are available with the online version of this article.

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Taxogenomics Resolves Conflict in the Genus *Rhodobacter*: A Two and Half Decades Pending Thought to Reclassify the Genus *Rhodobacter*

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The genus *Rhodobacter* is taxonomically well studied, and some members are model organisms. However, this genus is comprised of a heterogeneous group of members. 16S rRNA gene-based phylogeny of the genus *Rhodobacter* indicates a motley assemblage of anoxygenic phototrophic bacteria (genus *Rhodobacter*) with interspersing members of other genera (chemotrophs) making the genus polyphyletic. Taxogenomics was performed to resolve the taxonomic conflicts of the genus *Rhodobacter* using twelve type strains. The phylogenomic analysis showed that *Rhodobacter* spp. can be grouped into four monophyletic clusters with interspersing chemotrophs. Genomic indices (ANI and *d*DDH) confirmed that all the current species are well defined, except *Rhodobacter megalophilus*. The average amino acid identity values between the monophyletic clusters of *Rhodobacter* members, as well as with the chemotrophic genera, are less than 80% whereas the percentage of conserved proteins values were below 70%, which has been observed among several genera related to *Rhodobacter*. The pan-genome analysis has shown that there are only 1239 core genes shared between the 12 species of the genus *Rhodobacter*. The polyphasic taxonomic analysis supports the phylogenomic and genomic studies in distinguishing the four *Rhodobacter* clusters. Each cluster is comprised of one to seven species according to the current *Rhodobacter* taxonomy. Therefore, to address this taxonomic discrepancy we propose to reclassify the members of the genus *Rhodobacter* into three new genera, *Luteovulum* gen. nov., *Phaeovulum* gen. nov. and *Fuscovulum* gen. nov., and provide an emended description of the genus *Rhodobacter sensu stricto*. Also, we propose reclassification of *Rhodobacter megalophilus* as a sub-species of *Rhodobacter sphaeroides*.

Keywords: *Rhodobacter*, taxogenomics, *Rhodobacter sensu stricto*, gen. nov., *Rhodobacter* reclassification, phylogenomics, proposal of 3 new phototrophic genera, photosynthetic gene cluster

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ORIGINAL PAPER

Rhodobacter alkalitolerans sp. nov., isolated from an alkaline brown pond

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Abstract

An alkali-tolerant, Gram-stain-negative, motile, rod-to-oval-shaped, yellowish brown-colored, phototrophic bacterium, designated as strain JA916^T, was isolated from an alkaline brown pond in Gujarat, India. The DNA G + C content of the strain JA916^T was 65.1 mol%. Strain JA916^T grew well at pH 10. Respiratory quinone was Q-10 and major fatty acid was $C_{18:1}\omega7c$/$C_{18:1}\omega6c$, with significant quantities of $C_{15:0}2OH$ observed. Strain JA916^T shared the highest 16S rRNA gene sequence similarity with the type strains of *Rhodobacter johrii* (98.4%), followed by *Rhodobacter megalophilus* (98.3%), *Rhodobacter sphaeroides* (98.3%), *Rhodobacter azotoformans* (97.9%) and other members of the genus *Rhodobacter* (< 97%). 16S rRNA gene-based phylogenetic tree shows that strain JA916^T formed a distinct sub-clade with *Rhodobacter johrii, Rhodobacter megalophilus, Rhodobacter sphaeroides* and *Rhodobacter azotoformans*. Further, *rpoB*-based phylogenetic analysis showed lower similarity with closely related species (≤ 93.0%) of the genus *Rhodobacter*, which suggests that JA916^T is a novel species of the genus *Rhodobacter*. DNA–DNA hybridization values between strain JA916^T and related type strains were less than 40%. Phenotypic, chemotaxonomical and phylogenetic differences showed that strain JA916^T was distinct from other species of the genus *Rhodobacter*, suggesting strain JA916^T represents a new species of the genus for which the name *Rhodobacter alkalitolerans* sp. nov. is proposed. Type strain is JA916^T (= KCTC 15473^T = LMG 28749^T).

Keywords *Rhodobacter alkalitolerans* sp. nov. · Alkaline brown pond · *rpoB* gene

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA and *rpoB* gene sequences of the strains JA916^T are LN810645 and LT630399, respectively. DPD Taxon Number of the strain is TA00378.

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Introduction

The genus *Rhodobacter* belongs to the family *Rhodobacteraceae* in the class *Alphaproteobacteria*. Members of the genus *Rhodobacter* are Gram stain-negative facultative phototrophs (primarily phototrophic) and are widely distributed in oxic/anoxic zones of aquatic (Hansen and Imhoff 1985; Suresh et al. 2017), mud (Raj et al. 2013; van Niel 1944), sediment (Subhash and Lee 2016; Srinivas et al. 2008), agricultural field (Girija et al. 2010), estuarine (Venkata Ramana et al. 2008, 2009), sludge (Hiraishi et al. 1996) and marine (Srinivas et al. 2007) habitats. They play an important role as primary producers, nitrogen fixers and also help in bio-geochemical cycles. *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* have an extremely versatile metabolism and are considered as model organisms to carry out genetic, biophysical and metabolic studies. This study was carried out to locate the phylogenetic position of strain JA916^T, for which the name *Rhodobacter alkalitolerans* sp. nov. is proposed.

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Description of *Rhodobacter azollae* sp. nov. and *Rhodobacter lacus* sp. nov.

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Abstract

Three strains (JA826^T, JA912^T and JA913), which were yellowish brown colour, rod to oval shaped, Gram-stain-negative, motile, phototrophic bacteria with a vesicular architecture of intracytoplasmic membranes, were isolated from different pond samples. The DNA G+C content of the three strains was between 64.6 and 65.5 mol%. The highest 16S rRNA gene sequence similarity of all three strains was with the type strains of the genus *Rhodobacter sensu stricto* in the family *Rhodobacteraceae*. Strain JA826^T had highest sequence similarity with *Rhodobacter maris* JA276^T (98.5 %), *Rhodobacter viridis* JA737^T (97.5 %) and other members of the genus *Rhodobacter* (<97 %). Strain JA912^T had highest sequence similarity with *Rhodobacter viridis* JA737^T (99.6 %), *Rhodobacter sediminis* N1^T (99.3 %), *Rhodobacter capsulatus* ATCC 11166^T (98.8 %) and less than 97 % similarity with other members of the genus *Rhodobacter*. The 16S rRNA gene sequence similarity between strains JA826^T and JA912^T was 96.9 %. DNA–DNA hybridization showed that strains JA826^T and JA912^T (values among themselves and between the type strains of nearest members <44 %) did not belong to any of the nearest species of the genus *Rhodobacter*. However, strains JA912^T and JA913 were closely related (DNA–DNA hybridization value >90 %). The genomic distinction was also supported by differences in phenotypic and chemotaxonomic characteristics in order to propose strains JA826^T (=KCTC 15478^T=LMG 28758^T) and JA912^T (=KCTC 15475^T=LMG 28748^T) as new species in the genus *Rhodobacter sensu stricto* with the names *Rhodobacter lacus* and *Rhodobacter azollae*, respectively.

The genus *Rhodobacter* (*Rba*), proposed by Imhoff *et al.* [1] upon reclassification of the species of the genus *Rhodopseudomonas*, accommodated a few species of purple nonsulfur anoxygenic phototrophic bacteria namely, *Rhodobacter capsulatus* (type species), *Rhodobacter sphaeroides*, *Rhodobacter sulfidophilus* and *Rhodobacter adriaticus,* with a vesicular architecture of intracytoplasmic membranes. However, *Rba. sulfidophilus* and *Rba. adriaticus* were subsequently reclassified as *Rhodovulum sulfidophilum* and *Rhodovulum adriaticum*, respectively [2]. At the time of writing, the genus *Rhodobacter* comprises 13 recognized species names: *Rhodobacter aestuarii*, *Rhodobacter azotoformans*, *Rhodobacter blasticus*, *Rhodobacter capsulatus*, *Rhodobacter johrii*, *Rhodobacter maris*, *Rhodobacter megalophilus*, *Rhodobacter ovatus*, *Rhodobacter sphaeroides*, *Rhodobacter veldkampii*, *Rhodobacter vinaykumarii*, *Rhodobacter viridis* and the recently described *Rhodobacter sediminis* [3]. The genus *Rhodobacter* in the family *Rhodobacteraceae* includes a very heterogeneous assemblage of phototrophic bacteria with a large number of interspersing chemotrophic bacteria and their evolutionary relationships are not well established. According to the current taxonomy based on 16S rRNA gene sequence phylogenetic analysis, *Rhodobacter* species are grouped into five monophyletic clusters, each of which comprise one to seven species. Here, we propose to include the description of two new species of phototrophic bacteria affiliated to the genus *Rhodobacter sensu stricto*.

Strain JA826^T was isolated from a water sample of an industrially polluted fresh water lake near Hubbali, Karnataka, India (75° 20′ E, 15° 26′ N; sample pH 6.8). Strains JA912^T and JA913 were isolated from a fern, *Azolla filiculoides*, growing in a fresh water pond in Gujarat, India (GPS position: 72° 43′ E, 22° 47′ N; sample pH 6.0 and salinity 0.8 %). Both samples were serially diluted (10-fold dilution) and pour plated by using the wax overlay method [4] and incubated at 30 °C in the

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JA826^T, JA912^T, JA913 are LN835251, LN810641 and LN810642, respectively

Six supplementary figures and one supplementary table are available with the online Supplementary Material.

Reclassification of *Gemmobacter changlensis* to a new genus as *Cereibacter changlensis* gen. nov., comb. nov.

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We propose a new genus to accommodate the phototrophic bacterium *Gemmobacter changlensis* [Chen W. M., Cho, N. T., Huang, W. C., Young, C. C. & Sheu, S. Y. (2013) *Int J Syst Evol Microbiol* 63, 470–478] based on multiple strain analysis. Differences in the major diagnostic properties such as ability to grow phototrophically, the presence of internal photosynthetic membranes, the light harvesting complexes, fatty acids, carotenoids, bacterial chlorophylls, polar lipid composition and some other phenotypic properties warrant the creation of a new genus, designated *Cereibacter* gen. nov., to accommodate the phototrophic members of the genus *Gemmobacter*, as represented by the type species *Cereibacter changlensis* comb. nov.

A Gram-stain-negative, non-motile, oval to rod-shaped, psychrotolerant, phototrophic bacterium with vesicular-type intracytoplasmic membrane structures that contain bacteriochlorophyll-*a* and the spheroidene series of carotenoids was described as *Rhodobacter changlensis* (Anil Kumar *et al.*, 2007). However, based on 16S rRNA gene phylogenetic analysis, *Rhodobacter changlensis* was reclassified as *Catellibacterium changlense* (Zheng *et al.*, 2011) and subsequently as *Gemmobacter changlensis* (Chen *et al.*, 2013). The genera *Gemmobacter* (Rothe *et al.*, 1987) and *Catellibacterium* (Tanaka *et al.*, 2004) were described to accommodate non-phototrophic relatives of phototrophic alphaproteobacteria belonging to the family *Rhodobacteraceae*. The physiological groups of anoxygenic phototrophic bacteria are characterized primarily based on the light-driven energy process of anoxygenic photosynthesis and they are capable of phototrophic growth under anoxic conditions, and thus they are well-distinguished from their chemotrophic phylogenetic partners. Phototrophy is recognized as a genus-specific character (Imhoff & Caumette, 2004) and an important characteristic in differentiating the genera of phototrophs from those of chemotrophs (Subhash *et al.*, 2013c). Here, we have reinvestigated the phylogenetic affiliation of *G. changlensis* JA139^T, along with two additional strains, based on polyphasic taxonomy, and propose its reclassification into a new genus.

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JA139^T, JA749 and JA259 are AM399030, HE774677 and HF559005, respectively.

One supplementary figure is available with the online Supplementary Material.

Strains JA259 and JA749 were isolated from phototrophic enrichments of samples collected near Pangong lake [33° 56′ 58.10″ N 78° 25′ 28.34″ E (~4.498 km) and 34° 03′ 32.85″ N 78° 13′ 53.96″ E (~3.995 km), respectively] in the Indian Himalayas. Enrichment media, purification protocols and growth of the bacteria were as previously described (Lakshmi *et al.*, 2011a, b). For phototrophic growth, the cultures were grown in completely filled screw cap test tubes (10 × 100 mm) under phototrophic [light (2400 lx) anaerobic] conditions. Cultures were maintained on agar slants or as lyophilized cultures preserved at 4 °C.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G+C contents of the DNA of strains JA259 and JA749 (1379 bp) as determined by reversed-phase HPLC (Mesbah *et al.*, 1989) were 69.2 and 69.3 mol%, respectively. Well-isolated colonies were used for 16S rRNA gene amplification by using PCR master mix (GeNei) as described previously (Subhash *et al.*, 2013a). 16S rRNA gene sequencing was performed on a 3130xl Applied Biosystems ABI prism automated DNA sequencer, as described previously (Subhash *et al.*, 2013b). 16S rRNA gene sequences of strains JA259 (1379 bp) and JA749 (1373 bp) were subjected to BLAST search analysis on the EzTaxon-e server (Kim *et al.*, 2012). BLAST search analysis indicated that the two strains belonged to the genus *Gemmobacter* with *G. changlensis* JA139^T as their

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MICROBIOLOGY SOCIETY

Rhodomicrobium lacus sp. nov., an alkalitolerent bacterium isolated from Umiam lake, Shillong, India

Suresh G[1], Dhanesh Kumar[1], Jagadeeshwari Uppada[2], Sasikala Ch.[2,*] and Ramana Ch. V.[1,*]

Abstract

A Gram-stain-negative, motile, alkali-tolerant, swollen-rod shaped, reddish brown coloured, phototrophic bacterium designated as strain JA980^T, was isolated from freshwater sampled at Umiam lake, Shillong, India. Strain JA980^T grew well up to pH 9.0. Respiratory quinones were ubiquinone 10 and rhodoquinone 10. The major fatty acid was $C_{18:1}\omega7c/C_{18:1}\omega6c$ with minor amounts of $C_{18:0}$, $C_{16:0}$, $C_{18:0}$ 3-OH and $C_{16:0}$ 3-OH. Strain JA980^T contained bacteriochlorophyll-*a* and carotenoids of the spirilloxanthin series. The polar lipids of strain JA980^T comprised phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, an unidentified phospholipid, unidentified amino lipids (AL1,3,4,5) and an unidentified lipid (L1). Strain JA980^T had the highest (99.57%) 16S rRNA gene sequence similarity to the type strains of *Rhodomicrobium vannielii* ATCC17100^T and *Rhodomicrobium udaipurense* JA643^T. The genome of strain JA980^T was 3.88 Mbp with a DNA G+C content of 62.4 mol%. Based on the results of phylogenetic analyses, low *in silico* DNA–DNA hybridization values (33%), low (87%) average nucleotide identity results, chemotaxonomic characteristics and differential physiological properties, strain JA980^T could not be classified into either of the two recognized species of the genus *Rhodomicrobium*, suggesting that it represents a novel species, for which the name *Rhodomicrobium lacus* sp. nov. is proposed. The type strain is JA980^T (=KCTC 15697^T= MCC 3714^T= NBRC 113803^T).

The genus *Rhodomicrobium* belongs to the family *Hyphomicrobiaceae* in the class *Alphaproteobacteria*. Members of this genus are Gram-stain-negative, with distinct morphological features of cells having long prosthecae and a characteristic vegetative growth cycle [1]. They are facultative phototrophs (primarily phototrophic), grow on a wide range of aromatic hydrocarbons [2] and are widely distributed in various aquatic ecosystems [1, 3]. There are only two recognized [3] species of the genus *Rhodomicrobium* (*Rhodomicrobium vannielii* and *Rhodomicrobium udaipurense*). This study was carried out to locate the phylogenetic affiliation and taxonomic position of strain JA980^T, which was isolated from a fresh water lake in Shillong, India.

A sediment sample was collected from Umiam lake in Shillong, India (25° 68′ 18″ N 91° 92′ 53″ E). The lake at the time of sample collection had a pH of 7.5–8.5. The sample was subjected to enrichment of purple bacteria in modified Biebl and Pfennig's medium [4] under photoheterotrophic (2400 lux) conditions at 30 °C in a 40 ml transparent glass screw-cap bottle. After 6 days of incubation, reddish brown coloured turbidity was observed in the bottle. From this enrichment culture, strain JA980^T was purified anaerobically by repeated streaking on agar slants. Pure cultures were maintained on agar slants or as lyophilized cultures and preserved at 4 °C. Purified cultures were grown in completely filled screw-cap test tubes (10×100 mm) under photoheterotrophic conditions. Modified Biebl and Pfennig's medium [4] was used for purification and characterization.

Cell morphological features, such as division, motility, size and shape, were observed under phase contrast, confocal or scanning electron microscope (BH-2, Olympus/LSM880, Carl Zeiss/XL3O, Philips). Flagellar number and position were detected under transmission electron microscope (H-7500, Hitachi) using uranyl acetate as a negative stain.

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Keywords: Rhodomicrobium; phototrophic bacterium; new species.
Abbreviations: ANI, average nucleotide indentity; dDDH, digital DNA–DNA hybridization; ME, minimum-evolution; ML, maximum-likelihood; NJ, neighbour-joining.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the strain JA980^T is **LT934242**. The GenBank/EMBL/DDBJ accession for the whole genome shotgun sequence is **RZNF00000000**.
Six supplementary figures and one supplementary table are available with the online version of this article.

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MICROBIOLOGY SOCIETY

Paracoccus aeridis sp. nov., an indole-producing bacterium isolated from the rhizosphere of an orchid, *Aerides maculosa*

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Abstract

A Gram-stain-negative, non-motile, coccoid-shaped, catalase- and oxidase-positive, non-denitrifying, neutrophilic bacterium designated as strain JC501^T was isolated from an epiphytic rhizosphere of an orchid, *Aerides maculosa*, growing in the Western Ghats of India. Phylogenetic analyses based on the 16S rRNA gene sequence indicated that strain JC501^T belonged to the genus *Paracoccus* and had the highest levels of sequence identity with *Paracoccus marinus* KKL-A5^T (98.9 %), *Paracoccus contaminans* WPAn02^T (97.3%) and other members of the genus *Paracoccus* (<97.3 %). Strain JC501^T produced indole-3 acetic acid and other indole derivatives from tryptophan. The dominant respiratory quinone was Q-10 and the major fatty acid was $C_{18:1}\omega7c/C_{18:1}\omega6c$, with significant quantities of $C_{18:1}\omega9c$, $C_{17:0}$ and $C_{16:0}$. The polar lipids of strain JC501^T comprised phosphatidylglycerol, phosphatidylcholine, diphosphatidylglycerol, an unidentified glycolipid, two unidentified aminolipids, two unidentified lipids and four unidentified phospholipids. The genome of strain JC501^T was 3.3 Mbp with G+C content of 69.4 mol%. For the resolution of the phylogenetic congruence of the novel strain, the phylogeny was also reconstructed with the sequences of eight housekeeping genes. Based on the results of phylogenetic analyses, low (<85.9%) average nucleotide identity, digital DNA–DNA hybridization (<29.8%), chemotaxonomic analysis and physiological properties, strain JC501^T could not be classified into any of the recognized species of the genus *Paracoccus*. Strain JC501^T represents a novel species, for which the name *Paracoccus aeridis* sp. nov. is proposed. The type strain is JC501^T (=LMG 30532^T=NBRC 113644^T).

Bacteria residing in the rhizosphere of the plants play a vital role in the holistic development of the plant system [1–3]. Epiphytic orchid-associated bacteria have functional and ecological roles in the development of their host plant [4]. Epiphytes do not interact directly with the soil or its microbiota and thus constitute a unique system of ecology. Therefore, epiphytes have their own distinctive structural system for their sustenance, wherein they take up the nutrients and moisture from the atmosphere on the surface of the host plant aided by the microbial association [5, 6]. While investigating this unique diversity and its subsequent role in the development of the orchids, we have isolated strain JC501^T from the rhizosphere of an epiphytic orchid (*Aerides maculosa*). This strain belongs to the genus *Paracoccus* based on 16S rRNA gene sequence analysis. The genus *Paracoccus* was first described by Davis and his co-workers in 1969 [7] and belongs to the family '*Rhodobacteraceae*' of the class *Alphaproteobacteria* in the phylum *Proteobacteria*. There are more than 50 species of *Paracoccus* with validly published names (www.bacterio.net). Members have been isolated from environmental samples such as soil [8, 9], sediment [10, 11], water [12, 13], sludges [14, 15], foodstuffs [16], clinical specimens [17] and insects [18]. *Paracoccus halotolerans* [19], *Paracoccus salipaludis* [20], *Paracoccus fontiphilus* [13], *Paracoccus alimentarius* [16], *Paracoccus endophyticus* [21], *Paracoccus haematequi* [22] and *Paracoccus nototheniae* [23] are the valid names published during the year 2018–2019, while *Paracoccus jeotgali* [24] and *Paracoccus indicus* [25] are effective publications. Members of this genus are Gram-stain-negative, mostly non-motile and chemoorganotrophs [26]. Their major fatty acid is $C_{18:1}\omega7c$ and they are metabolically versatile [27]. The members of the genus *Paracoccus* have a genome size ranging from 2.9 to 5.6

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Keywords: *Paracoccus*; epiphytic orchid; Proteobacteria; indole.
Abbreviations: AIC, Akaike information criterion; ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; LCB, local collinear block; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NA, nutrient agar.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JC501^T is LT799401. The Whole Genome Shotgun project is SELD00000000. The genome sequence of *P. marinus* NBRC 100637^T is VJYZ00000000.
Five supplementary tables and ten supplementary figures are available with the online version of this article.

Emerging Concepts in Bacterial Taxonomy

1

Anusha Rai, Indu, N. Smita, G. Deepshikha, K. Gaurav, K. Dhanesh, G. Suresh, Ch. Sasikala, and Ch. V. Ramana

Abstract

Bacterial taxonomy has progressed over the years by virtue of the brisk and competent scientific developments. Ground-breaking molecular techniques have added an edge in the phylogenetic studies, resulting in the quality description of the taxa under studies. New avenues are rapidly developing whose validation has always been embraced and included, which will assist in resolution. It began with the simple application of objective procedures for classification, and now we have arrived at the genome-based taxonomy. This pedantic step has led to the meticulous examination and served to reconcile certain conflicts of the status of the taxa. This field is dynamic and is exploring more options like proteomics and metabolomics in gaining more insights into the lineal heritage. Even though there has been a significant change and addition, there is an ever-growing need for a comprehensive study, which would thread all the attributes together into one functional unit of classification. In this review, we examine the paradigm shift from traditional taxonomy to integrated taxonomy useful in the characterisation of bacteria which in addition aids in the identity of biotechnological targets.

Keywords

Bacterial taxonomy · Polyphasic · Phylogenomics · Integrated taxonomy · Average nucleotide sequence index (ANI)

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MICROBIOLOGY SOCIETY

Afifella aestuarii sp. nov., a phototrophic bacterium

AQ1 Sailaja Buddhi[1], Suresh G.[2], Deepshikha Gupta[2], Sasikala Ch.[1,*] and Ramana Ch. V.[2,*]

Abstract

An oval- to rod-shaped, motile, Gram-stain-negative, oxidase-positive, catalase-negative, pink-coloured phototrophic bacterium (designated as strain JA968^T) was isolated from an estuary near Pata, Gujarat, India. Cells had an intracytoplasmic membrane architecture as lamellae and divided by budding. Strain JA968^T had bacteriochlorophyll-*a* and spirilloxanthin series carotenoids as photosynthetic pigments. The strain exhibited photolithoautotrophic, photoorganoheterotrophic and chemoorganoheterotrophic growth modes and required thiamine as a growth factor. Strain JA968^T had $C_{18:1}\omega7c/C_{18:1}\omega6c$ as the predominant fatty acid with ubiquinone-10 (Q-10) and menaquinone-10 (MK-10) forming the quinone composition. The genomic DNA G+C content of the strain was 63.5 mol%. Pairwise comparison of 16S rRNA gene sequences showed that strain JA968^T was highly similar to *Afifella marina* DSM 2698^T (99.9 %) and *Afifella pfennigii* DSM 17143^T (98.4 %). The average nucleotide identity values were 92% between strain JA968^T and *A. marina* DSM 2698^T, and 78% between strain JA968^T and *A. pfennigii* DSM 17143^T. The digital DNA–DNA hybridization values between strain JA968^T and *A. marina* and *A. pfennigii* were 49 and 19%, respectively. The genomic distinction was also supported by differences in phenotypic and chemotaxonomic characteristics. We propose that strain JA968^T represents a new species of the genus *Afifella* with the name Afifella *aestuarii* sp. nov. The type strain is JA968^T (=KCTC 15634^T=NBRC 113338^T).

The genus *Afifella* of the family *Rhodobiaceae* proposed by Urdiain *et al.* [1] accommodates two species with validly published names (*Afifella marina* and *Afifella pfennigii*) of purple non-sulfur anoxygenic phototrophic bacteria. These members are phototrophic, commonly isolated from marine habitats, require NaCl for optimum growth and multiply by budding [2–4]. Through this study, we propose strain JA968^T as representing a new species of this genus. It was isolated from an estuarine sample collected from Pata, Gujarat, India (21° 218′ N; 69° 931′ E). The sample had a pH of 7.0 and salinity of 0.5%.

The sample was kept for enrichment in a media described previously [5] with pyruvate (22 mM) as the carbon source/electron donor in fully filled (45 ml) screw-capped glass bottles and incubated at 2400 lux (light), 28 °C for 5 days. Strain JA968^T was purified by repeated streaking on agar slants. Purified cultures were maintained on agar slants or as lyophilized cultures preserved at 4 °C. Further characterization of strain JA968^T was carried out in the medium described previously [5].

Genomic DNA was extracted using the nucleopore gDNA bacterial/fungal mini kit as per the manufacturer's instructions. The same DNA was used for 16S rRNA gene amplification and whole-genome sequencing. A nearly full-length (1463) 16S rRNA gene sequence of strain was achieved using the protocol of Divyasree *et al.* [6]. Whole-genome sequencing was outsourced to Agrigenome labs Pvt. Ltd, Kochi, India. Whole-genome DNA sequencing was carried out using the Illumina Hiseq-4000 platform. The sequenced data was assembled using VELVET 1.2.10 *de novo* assembly software.

The results of 16S rRNA gene sequence BLAST search analysis on the EzBioCloud database [7] indicated that the strain shared highest similarity with the type strains of the genus *Afifella*. Therefore, the 16S rRNA gene sequences of strain JA968^T, *A. marina* DSM 2698^T and *A. pfennigii* DSM 17143^T were derived from whole-genome sequencing and used for phylogenetic analysis. Genome sequences of *A. marina* and *A. pfennigii* were obtained from NCBI (www.ncbi.nlm.nih.gov/

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Keywords: *Afifella*; photosynthetic; alphaproteobacteria.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA968^T is LT622289. The accession number for the draft genome sequence of strain JA968^T is SAUF00000000.
One supplementary table and nine supplementary figures are available with the online version of this article.

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ORIGINAL CONTRIBUTION

Organic/inorganic hybrid nanocolloids of water dispersible polyurethanes with antibacterial activity

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Abstract We report an in-situ synthetic strategy to prepare water dispersible polyurethane (WDPU) from an organic/inorganic hybrid nanocolloids in which the organic component is dinitrobenzene-modified hydroxyl-terminated polybutadiene (HTPB-DNB)-based polyurethane and the inorganic part is TiO_2 (core)–SiO_2 (shell) nanoparticles. The shape, size, and polydispersity of the hybrid nanocolloids are determined using microscopic and light scattering studies and are found to be slightly dependent on the loading of core–shell nanofillers. FT-IR and solid-state NMR studies prove the presence of the interaction between the carbonyl functionality of the urethane linkage and the SiO_2 present in the shell part of the particles, and this interaction is found to be the driving force for the formation of stable nanocolloids. The films, obtained from the nanocolloids of WDPU upon curing, display better thermal and mechanical properties, and also, these properties increase with increasing loading of nanofillers. Higher tensile strength and Young modulus are obtained in WDPU nanocolloids films compared to pristine WDPU films. The antibacterial activity of WDPU nanocolloids was studied on *Staphylococcus aureus* (Gram-stain-positive bacterium) and *Escherichia coli* (Gram-stain-negative bacterium) displayed significantly higher zone of inhibition in both the cases, indicating the potential use of these nanocolloids in antibacterial coating.

Keywords Hybrid nanocolloids · Water dispersible polyurethane · Butadiene diol · Core–shell nanoparticles · Antibacterial activity

Introduction

The water dispersible polyurethanes (WDPUs) are widely used for coating applications because of environmental consideration, and hence, many researchers are putting their efforts to prepare the WDPUs with improved properties [1–6]. Among these, thermo-responsive amphiphilic polyurethane gels are more interesting and can be used as biodegradable carriers for drugs and as cell printing materials near body temperature [5, 6]. Subramani et al. developed polyurethane dispersion from water dispersible/reducible anionic MEKO, DMP, and ε-caprolactam-blocked aromatic diisocyanates [1]. The synthetic processes and chemistry of waterborne polyurethanes were discussed by Karl-Ludwig Noble [2]. The article also discussed the importance of WDPU for environmental concerns. Ma XY and Zhang WD reported a novel waterborne polyurethane/flower-like ZnO nanowhisker composite and studied their antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* [3]. Despite many advantages of WDPUs, there are several disadvantages such as the thermal stability, insolubility, and mechanical properties which need to be addressed [3, 7–9]. In addition, hydrophobicity and antibacterial activity of the cured WDPU polymeric films would be of very useful since they are good for coating applications. Several researchers have used nanoscale inorganic fillers

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MICROBIOLOGY SOCIETY

Chryseobacterium salipaludis sp. nov., isolated at a wild ass sanctuary

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Abstract

A Gram-stain-negative, rod-shaped, non-motile, aerobic bacterium was isolated from a sediment sample obtained from a wild ass sanctuary in Gujarat, India. The strain designated JC490^T was oxidase- and catalase-positive. The 16S rRNA gene sequence analysis and sequence comparison data indicated that strain JC490^T was a member of the genus *Chryseobacterium* and was closely related to *Chryseobacterium jeonii* AT1047^T (96.4 %) and with other members of the genus *Chryseobacterium* (<96.3 %). The DNA G+C content of strain JC490^T was 34 mol%. Strain JC490^T had phosphatidylethanolamine, two unidentified aminolipids, two unidentified phospholipids and five unidentified polar lipids. Menaquinone-6 was the only respiratory quinone found. Iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH were the major fatty acids of strain JC490^T. On the basis of physiological, genotypic, phylogenetic and chemotaxonomic analyses, it is concluded that strain JC490^T constitutes a novel species of the genus *Chryseobacterium,* for which the name *Chryseobacterium salipaludis* sp. nov. is proposed. The type strain is JC490^T (=KCTC 52835^T=LMG 30048^T).

The genus *Chryseobacterium*, which belongs to the family *Flavobacteriaceae*, was first established by Vandamme *et al.* [1]. Subsequently, the genus expanded rapidly to encompass 100 species (www.bacterio.net/chryseobacterium.html), isolated from various environments, which include soils [2], clinical samples [3], fresh water [4], sewage and wastewater [5], plants [6], fish [7], and meat [8]. The typical characteristics of the genus *Chryseobacterium* include the presence of an aerobic type of metabolism, branched-chain fatty acids (iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH) as the major fatty acids, phosphatidylethanolamine as a major polar lipid, menaquinone-6 (MK-6) as the characteristic respiratory quinone and production of flexirubin-type pigments [9–14]. During our studies on the bacterial diversity of various salt marsh habitats of India, strain JC490^T was isolated from a sediment sample and characterized by a polyphasic taxonomic approach.

Strain JC490^T was isolated from a sediment sample of a salt marsh which is in a wild ass sanctuary, at Dhrangadhra, Gujarat, India (22° 98′ N 71° 47′ E). One gram of sample was serially diluted [in 0.8 % (w/v) NaCl] and plated on nutrient agar (NA; HiMedia M001). Several different colony morphologies were observed on the plates upon incubation at 30 °C for 2 weeks and a yellow colony was purified by repeated streaking and designated as strain JC490^T. Strain JC490^T grew well on NA and routine sub-culturing of the strain was done on the same medium at 35 °C for 48 h. Pure cultures were lyophilized and preserved at 4 °C for further use.

DNA was extracted and purified by using a Qiagen genomic DNA extraction kit. The 16S rRNA gene was amplified by using the F27 and R1492 primers. The purified PCR product was ligated to the pTZ57R/T (Thermo Fisher Scientific) vector and cloned according to the manufacturer's instructions and 16S rRNA gene sequencing was carried out as described previously by Divyasree *et al.* [15]. Basic Local Alignment Search Tool (BLAST) search analysis of the 16S rRNA gene sequence of strain JC490^T (1481 nt) using the EzBioCloud database [16] showed highest sequence similarity to *Chryseobacterium jeonii* AT1047^T (96.4 %) and with other members of the genus *Chryseobacterium* (<96.3 %). The 16S rRNA gene sequences were aligned using the Silva Incremental Aligner (SINA; www.arb-silva.de) and Molecular Evolutionary Genetics Analysis 7 (MEGA7) [17] software was used for phylogenetic analyses. Distances were calculated by using Kimura's two-parameter method [18] in a pair-wise deletion procedure. Neighbour-joining (NJ), maximum likelihood (ML), minimum evolution (ME) and maximum parsimony (MP) methods in the MEGA7 software were used to reconstruct phylogenetic trees and a combined phylogenetic

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Keywords: *Chryseobacterium*; new species.
The GenBank/EMBL/DDBJ accession number for the 16SrRNA gene sequence of strain JC490^T is LT795113.
Two supplementary figures are available with the online version of this article.

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Salinicoccus amylolyticus sp. nov., isolated from a saltern

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A Gram-stain-positive coccus, strain JC304^T, was isolated from a saltern of Nari along the Bhavnagar Coast, Gujarat, India. The 16S rRNA gene sequence analysis and sequence comparison data indicated that JC304^T represented a member of the genus *Salinicoccus* and was most closely related to *Salinicoccus roseus* 9^T (99.6 %), *Salinicoccus luteus* YIM 70202^T (97.0 %), *Salinicoccus hispanicus* J-82^T (97.0 %) and the remaining species of the genus *Salinicoccus* (<97 %). Genome relatedness based on DNA–DNA hybridization of JC304^T with the type strains of the most closely related species was less than 46 % and the ΔT_m was >5 °C indicating that the strain represents a novel species of the genus *Salinicoccus*. Independent and concatenated phylogenetic analysis of *recA*/*fusA* gene translated product showed a clear distinction of JC304^T from its phylogenetic neighbors. Diphosphotidylglycerol, phosphatidylglycerol, an unidentified glycolipid and three unidentified lipids (L1, L2 and L3) were the polar lipids of JC304^T. Iso-$C_{15:0}$ and anteiso-$C_{15:0}$ were the major (>10 %) fatty acids in strain JC304^T. The cell-wall amino acids were L-lysine and D-glycine. Hopanoids were not detected. The major isoprenoid quinone was menaquinone (MK-6). The DNA G+C content of JC304^T was 48 mol%. On the basis of physiological, genotypic, phylogenetic and chemotaxonomic analyses, strain JC304^T is considered to represent a novel species of the genus *Salinicoccus*, for which the name *Salinicoccus amylolyticus* sp. nov. is proposed. The type strain is JC304^T (=KCTC 33661^T=LMG 28757^T).

The genus *Salinicoccus* was first proposed by Ventosa *et al.* (1990) and belongs to the family *Staphylococcaceae*, a member of the phylum Firmicutes. Species of the genus *Salinicoccus* have been isolated from salt mines (Chen *et al.*, 2007, 2009; Franca *et al.*, 2006; Wang *et al.*, 2008; Ramana *et al.*, 2013), soda lake (Zhang *et al.*, 2002), fermented foods (Aslam *et al.*, 2007; Pakdeeto *et al.*, 2007; Jung *et al.*, 2010), desert soil (Zhang *et al.*, 2007), sea water (Qu *et al.*, 2012), waste waters (Amoozegar *et al.*, 2008) and rhizosphere soil (Kämpfer *et al.*, 2011). The genus *Salinicoccus* includes 15 species with validly published names (http://www.bacterio.net/salinicoccus.html). Members of the genus *Salinicoccus* are moderately halophilic, aerobic, Gram-stain-positive cocci, which are chemotaxonomically characterized by having menaquinone-6 (MK-6) as the predominant isoprenoid quinone, a cell wall peptidoglycan type based on L-Lys-D-Gly_5 and a DNA G+C content of 46–51 mol% (Ventosa *et al.*, 1992). '*Salinicoccus kekensis*' (Gao *et al.*, 2010) and '*Salinicoccus salitudinis*' (Chen *et al.*, 2007) are later additions to the genus whose names have not been validly published at the time of writing.

During our studies on bacterial diversity of hypersaline habitats of India, strain JC304^T was isolated from soil collected from a salt pan at Nari along the Bhavnagar Coast, Gujarat, India (GPS position of sampling site: 21°78′ N 72°08′ E) after serial dilution (in 0.8 % w/v NaCl) and plating on marine agar 2216 (MA, pH 9; Himedia). Several different colony morphologies were observed on plates that had been incubated at 30 °C for 2 weeks. A pink–red colored colony was purified by repeated streaking and the the purified isolate was designated as JC304. On MA medium, colonies of JC304^T were circular, smooth, slightly elevated, approximately 2 mm in diameter and pink–red. JC304^T cells were observed after Gram-staining (protocol of Norrell & Messley, 1997) using a BH-2 phase contrast microscope (Olympus). Cells were coccus shaped (1.0–2.5 μm; Fig. S1, available in the online

Abbreviation: MLSA, multilocus sequence analysis.

The Genbank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strain JC304^T is LN866629.

One supplementary table and seven supplementary figures are available with the online Supplementary Material.

Taxonomy of the genus Rhodobacter

by Suresh G

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