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Research Letter

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Diversity of *Rhodopirellula* and related planctomycetes in a North Sea coastal sediment employing *carB* as molecular marker

Marina Žure[1], Colin B. Munn[2] and Jens Harder[1,*]

[1]Dept. of Microbiology, Max Planck Institute for Marine Microbiology, D-28359 Bremen, Germany and [2]School of Marine Sciences and Engineering, University of Plymouth, Plymouth PL4 8AA, UK

*Corresponding author: Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Tel: +49-421-2028-750; Fax: +49-421-2028-590; E-mail: jharder@mpi-bremen.de

One sentence summary: Cultivation-independent identification of the diversity of *carB* genes in a sediment sample within the genus *Rhodopirellula*, a step towards a true identification of species numbers in natural samples.

Editor: Andrew Millard

ABSTRACT

Rhodopirellula is an abundant marine member of the bacterial phylum *Planctomycetes*. Cultivation studies revealed the presence of several closely related *Rhodopirellula* species in European coastal sediments. Because the 16S rRNA gene does not provide the desired taxonomic resolution to differentiate *Rhodopirellula* species, we performed a comparison of the genomes of nine *Rhodopirellula* strains and six related planctomycetes and identified *carB*, coding for the large subunit of carbamoylphosphate synthetase, as a suitable molecular marker. In this study, we investigated the diversity of *Rhodopirellula* in coastal intertidal surface sediments of Sylt island, North Sea, using the 16S rRNA and *carB* genes as molecular markers. The *carB* clone and pyrosequencing libraries revealed the presence of 12 species of *Rhodopirellula* and of 66 species in closely related undescribed genera, a diversity that was not detected with a 16S rRNA gene library. This study demonstrates that the *carB* gene is a powerful molecular marker for detecting *Rhodopirellula* species in the environment and may be used for the taxonomic evaluation of new strains.

Keywords: *Rhodopirellula*; 16S rRNA gene; *carB* gene; diversity; molecular marker

INTRODUCTION

The genus *Rhodopirellula* belongs to the widespread bacterial phylum *Planctomycetes* in the *Planctomycetes–Verrucomicrobia–Chlamydiae* superphylum (Wagner and Horn 2006). The planctomycetes possess phenotypic characteristics unusual for the domain *Bacteria*, including reproduction by budding, and an intracellular membrane-bounded compartmentalization, the extent of which varies between species (Fuerst and Sagulenko 2011; Speth, van Teeseling and Jetten 2012; Santarella-Mellwig *et al.* 2013). Recently, cyro-transmission electron microscopy and cyro-electron tomography studies provided evidence for a peptidoglycan layer (Jeske *et al.* 2015; van Teeseling *et al.* 2015). *Planctomycetes* are abundant and highly diverse and have been proposed to contribute to the global carbon cycle by turnover of complex carbohydrates in marine sediments and marine snow (Glöckner *et al.* 2003). *Planctomycetes* include free-living as well as attached-living organisms.

Members have been associated with phytoplankton blooms (Morris, Longnecker and Giovannoni 2006; Pizzetti *et al.* 2011) marine snow particles (DeLong, Franks and Alldredge 1993; Gade *et al.* 2004; Fuchsman *et al.* 2012) and in association with several eukaryotic organisms such as prawns and sponges (Fuerst *et al.*

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1997; Pimentel-Elardo *et al.* 2003; Izumi *et al.* 2013). Several *Planctomycetes* belonging to new genera or new *Rhodopirellula* species were isolated from the microbial community on macroalgae (Bengtsson and Øvreås 2010; Lage and Bondoso 2011). Winkelmann and Harder (2009) isolated 70 strains from European seas, which affiliated according to 16S rRNA gene analysis with the strain *Rhodopirellula baltica* SH1^T. In a multilocus sequence analysis and in combination with DNA–DNA hybridization (DDH) experiments, those isolates were arranged into 13 operational taxonomic units (OTUs) suggesting a high diversity of *Rhodopirellula*, which could not be deduced using the 16S rRNA gene solely as a marker gene (Winkelmann *et al.* 2010).

The 16S rRNA gene is a powerful marker for classification of microorganisms and the taxonomic resolution of this gene enables the differentiation of genera, but not of closely related species in a genus (Yarza *et al.* 2014). Therefore, the bacterial species definition can never be based solely on sequence similarity of 16S rRNA genes. Two organisms with almost identical 16S rRNA gene sequences can still be recognized as two different species based on DDH (Fox, Wisotzkey and Jurtshuk 1992). Stackebrandt and Ebers (2006) recommended that above a value between 98.7 and 99% in the 16S rRNA gene sequence similarity, DDH would be necessary. With the advent of rapid genome sequencing, DDH is being replaced by determination of the average nucleotide identity (ANI) of shared genes between two strains (Richter and Rosselló-Móra 2009). Konstantinidis and Tiedje (2005) showed that ANI values of 94–95% correspond to 70% similarity in DDH experiments, the current standard of the species definition. Besides the 16S rRNA gene, other molecular markers can be used to resolve bacterial phylogenetic relationships. Recently, the *rpoB* gene, coding for the beta subunit of the RNA polymerase, has been used for the taxonomic affiliation of *Planctomycetes* strains (Bondoso, Harder and Lage 2013).

The aim of this study was to investigate the diversity of *Rhodopirellula* in sandy intertidal sediments of Sylt island, Germany, using as molecular markers 16S rRNA and *carB* genes, the latter coding for the large subunit of carbamoylphosphate synthetase. The *carB* gene was selected based on a comparison of genomes of nine *Rhodopirellula* strains (Glöckner *et al.* 2003; Klindworth *et al.* 2014; Richter *et al.* 2014a,b; Richter-Heitmann *et al.* 2014; Wegner *et al.* 2014) and six related planctomycetes following the requirements for candidate genes used in a species prediction (Stackebrandt *et al.* 2002).

MATERIALS AND METHODS

Sampling and DNA extraction

Samples from the upper 2 cm of sandy coarse sediment were obtained from two locations, Hausstrand beach (55.01518 N, 8.43814 E) and Weststrand beach (55 03840 N, 8 38490 E) on the coast of Sylt island, Germany. Samples were collected into 15 ml falcon tubes and frozen at −80°C till further processing. Genomic DNA was extracted from 500 mg of sediment using FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to manufacturer instructions. The quantity and quality of extracted DNA was determined with a NanoDrop 100 Spectrophotometer (Thermo Fisher Scientific, USA) followed by agarose gel electrophoresis and ethidium bromide (EtBr) staining.

PCR amplification of 16S rRNA gene

The general bacterial 16S rRNA gene forward primer 8–27F (Juretschko *et al.* 1998) and the planctomycete-specific reverse primer PLA886 (Neef *et al.* 1998) were used to amplify partial 16S rRNA gene in 30 μl of PCR mixture containing 10–15 ng of genomic DNA, 3.3 μM of each primer and 15 μl of GoTaq® Hot Start Colorless Master Mix (Promega, USA). The template DNA was denatured for 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 65°C, 3 min at 72°C and a final extension of 10 min at 72°C.

Design and PCR amplification of *carB* gene

Alignments of functional genes that were present in single copy in nine *Rhodopirellula* and six other *Planctomycetes* genomes (*Blastopirellula marina, Gemmata obscuriglobus, Gimesia* (formerly *Planctomyces*) *maris, Planctopirus* (formerly *Planctomyces*) *limnophilus, Pirellula staleyi and Kuenenia stuttgartiensis*) were used to identify suitable marker genes. Criteria for marker gene regions included a unique PFAM model, an annotated function, two conserved regions flanking 500–800 bases of high variability, and primer sites conserved in all nine *Rhodopirellula* genome and very different in the other planctomycetal genomes. Genes were selected after manual alignment inspection of 373 candidate genes, and primers were designed using the conservation plots. For the *carB* gene, a forward degenerated primer at the position 2095–2114 (5′-GCHCGBAACATGGAMGAAGC-3′) and a reverse degenerated primer at the postion 2827–2808 (5′-CVGCGAKTTGGCTYTTKGCR-3′) were highly specific for *Rhodopirellula* strains and used to generate 693 bp long *carB* amplicons. The PCR mixture contained 10–15 ng of genomic DNA, 3.3 μM of each carB primer and a 2x PCR master mix (Promega, USA) in 25 μl volume. The template DNA was denatured for 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, 3 min at 72°C and a final extension of 10 min at 72°C.

Gene library construction and sequencing of 16S rRNA gene and *carB* amplicons

16S rRNA gene and *carB* PCR amplicons were purified on Sephadex G-50 Superfine columns (Amersham Biosciences AB, Uppsala, Sweden) and ligated into the vector pCR4 applying the TOPO® TA cloning (Invitrogen, USA). Inserts were sequenced using the BigDye Terminator v3.1 Cyle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an ABI PRISM 3130xl Genetic Analyzer.

454 pyrosequencing of *carB* amplicon

In addition to the clone library, a *carB* amplicon from a Hausstrand sample was analyzed by 454 pyrosequencing. PCR amplification was performed in two steps. First, PCR amplicons were obtained according to the aforementioned protocol with 20 cycles of amplification. The PCR products were purified with the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) and 1 μl of purified PCR amplicon was used for a second PCR amplification with fusion primers according to the aforementioned protocol for 20 cycles. Fusion primers contained in front of the *carB* primers a linker required for 454 sequencing, forward fusion primer (5′- GATGGCCATTACGGCC -GCHCGBAACATGGAMGAAGC-3′), and reverse fusion primer (5′-GGTGGCCGAGGCGGCCACACGT- CVGCGAKTTGGCTYTTKGCR-3′). The amplicon was purified and sequenced on a 454 GS FLX sequencer by the Max Planck-Genome-centre Cologne (http://mpgc.mpipz.mpg/home/).

Analysis of 16S rRNA gene and *carB* clone library sequences

16S rRNA gene and *carB* sequences were analyzed with Sequencing Analysis 5.2 (Applied Biosystems, Carlsbad, CA, USA) and truncated within Sequencer 4.6 (Gene Codes, Michigan, USA). The 16S rRNA gene sequences were aligned in ARB software (Ludwig *et al.* 2004) using the SINA aligner (Pruesse, Peplies and Glöckner 2012). Manual refinement was conducted by comparison with their closest relatives. The *carB* sequences were first translated into protein sequences and visually inspected for the presence of stop codons. Translated sequences were aligned with MAFFT (Katoh, Asimenos and Toh 2009), and the nucleotide sequence alignment was generated according to the protein alignment. Distance matrices of 16S rRNA gene and *carB* sequences were calculated using the neighbor-joining method as implemented in ARB. OTU clustering was performed based on those matrices using the software Mothur v 1.29.1 (Schloss *et al.* 2009). Representative OTU sequences were used to calculate phylogenetic maximum likelihood (ML) trees in ARB using RAxML 7.0.4 (Stamatakis 2006) with 50% minimal similarity filter.

Analysis of *carB* pyrosequencing reads

Pyrosequencing reads were first processed in Mothur. The analysis included removal of primer sequences, quality control to remove sequences with ambiguous nucleotides, denoising and removal of chimeric sequences using the UCHIME algorithm (Edgar *et al.* 2011). Sequences were translated, inspected for stop codons and aligned with MAFFT. The corresponding nucleotide sequence alignment was generated according to the protein alignment. A distance matrix was calculated using the neighbor-joining method as implemented in ARB, and OTU clustering was performed based on that matrix using the software Mothur. OTU representatives of 454 reads were aligned together with the OTU representative sequences of the *carB* clone libraries and the alignment confidence scores were assessed using the GUIDANCE algorithm (Penn *et al.* 2010b) implemented in the GUIDANCE web server (Penn *et al.* 2010a). Sequences below the confidence score of 0.826 were removed from the analysis. The 454 reads were then placed in the reference ML *carB* tree using the evolutionary placement algorithm (Berger, Krompass and Stamatakis 2011) implemented in RAxML 8 (Stamatakis 2014).

Accession numbers

The 16S rRNA and *carB* nucleotide sequences of environmental origin and of strains obtained in this study were deposited in the Genbank under accession numbers KT283954–KT284072, KT284073–KT284305, KT284306–KT284310, respectively. The *carB* gene 454 sequencing SFF file was deposited to the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov) under accession number SRR2097334.

RESULTS

Analysis of 16S rRNA gene clone library

The clone libraries from both sample sites yielded 158 sequences, of which 119 affiliated with *Planctomycetes*. The 119 planctomycetal sequences were aligned covering the 16S rRNA gene positions 57–906 (numbering related to *Escherichia coli* sequence) and grouped into OTUs. Genus-related threshold of 95% (Roselló-Móra and Amann 2001) resulted in 63 OTUs, whereas species-related threshold of 97% (Roselló-Móra and Amann 2001) and 99% (Stackebrandt and Ebers 2006) resulted in 73 and 90 OTUs, respectively. The phylogenetic analysis placed seven OTUs at the 97% and nine OTUs at the 99% threshold closer to the genus *Rhodopirellula* than to its next validly described relative *Blastopirellula*, but none of the clone sequences affiliated closely with the species in the genus *Rhodopirellula* (Fig. 1). OTUs with the closest affiliation to *Rhodopirellula* formed a group with the strain SM50, which represents a novel genus based on a gene identity of 93.7% for the complete 16S rRNA gene (Winkelmann *et al.* 2010) and is tentatively named '*Rhodopilula apulia*'.

Correlation between *carB* gene and ANI

To resolve intra- and interspecies relationship and to define the species threshold for the partial *carB* gene (693bp) used in this study, the pairwise similarity values of the *carB* gene were plotted against the ANI values between each pair of genomes (Fig. 2). The ANIs were determined by using the *in silico* DDH method of the JSpecies software (Richter and Rosselló-Móra 2009). Strains used for the calculation and *carB* similarities of strains to *R. baltica* SH1^T are shown in Table 1. Genome analyses of four strains of *R. baltica* and two strains of the related species '*Rhodopirellula europaea*' established an intraspecies similarity of the amplicon region of at least 97.4%. The pairwise interspecies ANI for the two species was 88% for the common genes in the genomes and less than 93.1% for the *carB* amplicon. Thus, a threshold of 95% was well suited to separate closely related *Rhodopirellula* species. *Blastopirellula marina* and *P. staleyi* had higher sequence similarity (81.1%) than some strains within *Rhodopirellula* e.g. '*R. europaea*' 6C and '*R. maiorica*' SM1 (77.7%). Also strain SM50 of the genus '*Rhodopilula*' had a similarity of 77.1% to *R. rubra* SWK7; thus, a genus threshold could not be established solely on the basis of *carB* partial gene sequences.

The *carB* sequences of the 454 pyrosequences covered an alignment of 204 bp in length. In contrast to the 693 bp alignment of Sanger sequences, this *carB* region was slightly higher conserved, resulting in a species border threshold of 97%.

Analysis of *carB* sequences

Two *carB* clone libraries gave 233 sequences which clustered into 48 OTUs on a 95% similarity threshold. The representative sequences of the OTUs and the *carB* sequences of *Planctomycetes* strains were used for the phylogenetic analysis (Fig. 3). Strains and corresponding *carB* gene accession numbers are listed in Table 1. The tree revealed that the majority of the OTUs were more closely related to *Rhodopirellula* than to *Blastopirellula*. We detected one OTU that affiliated with *R. baltica* SH1^T with 99.6% similarity and one OTU that clustered with '*R. maiorica*' SM1 with 96.7% similarity.

A deeper insight into the species diversity was obtained by 454 pyrosequencing of a *carB* amplicon. After preprocessing, the dataset included 7763 reads with a length of 204 bp. The *carB* sequences of the genomes had indicated for the amplicon size of 204 bp a higher species border threshold of 97%. Applying this threshold, the reads clustered in 290 OTUs, of which 157 OTUs were singletons. Chao1, a conservative richness estimator, predicted the presence of 650 OTUs (548–792). After removing singletons and sequences with alignment confidence score below 0.826, 95 OTUs remained and were added to an already constructed ML tree of *carB* clone and strain sequences (Fig. 3). Considering the relatedness to the *Rhodopirellula* strain sequences, *carB* sequences obtained in this study clustered in four groups.

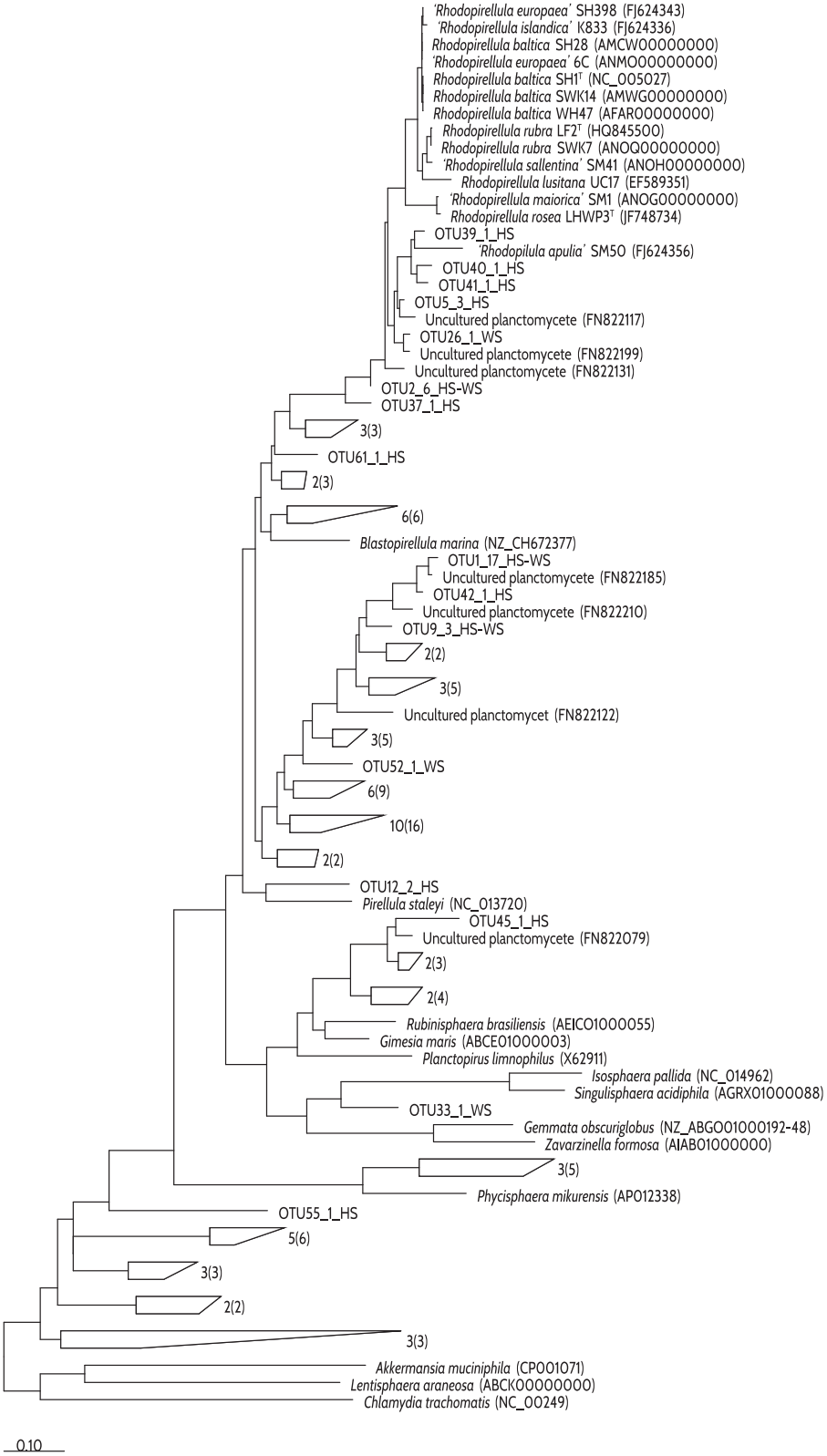

Figure 1. Phylogenetic ML tree of 16S rRNA gene showing the affiliation of representative sequences of clone library OTUs clustered at 97% similarity, cultured *Planctomycetes* strains and uncultured planctomycetes (Pizzetti *et al.* 2011). Labeling: OTU representatives, OTU (number of OTU_number of sequences in the OTU_sample site); groups (number of OTUs in the group) (total number of sequences). *Verrucomicrobia*, *Lentisphaerae* and *Chlamydiae* sequences were used as outgroup. Scale bar represents 0.1 substitutions per 100 nucleotides.

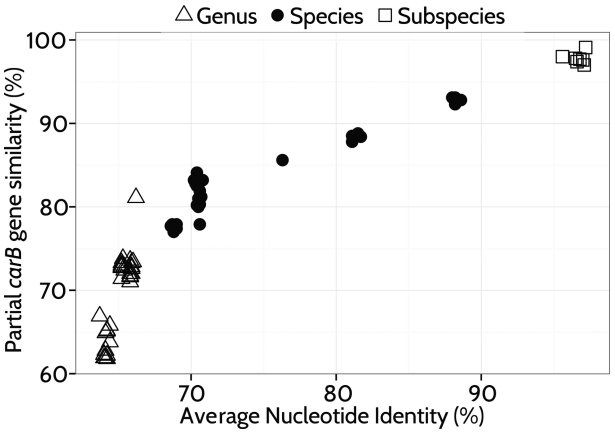

Figure 2. Scatter plot representing the pairwise correlation between the partial *carB* gene sequence (690 bp) similarity and the ANI. Each data point represents a pairwise sequence comparison of the *carB* gene plotted against the ANI between two strains representing one species (square), two species within a genus (filled circle) or two genera (triangle). Strains used for the calculation are listed in Table 1.

Group B contained cultured *Rhodopirellula* strains, clone library sequences of *R. baltica* SH1^T and '*R. maiorica*' SM1 as well as pyrosequencing reads of *R. baltica*, *R. rosea*, '*R. europaea*', *Rhodopirellula* sp. CS14 and '*R. islandica*' K833. In absolute numbers, group B included 154 reads and 13 clone sequences. In addition to known strains, phylogenetic analysis of the group B suggested a presence of six so far uncultured *Rhodopirellula* species. Pairwise identities within group B were 73–100% between members of the group, 72–75% to '*R. apulia*' SM50 (group C) and 67–73% to *B. marina* and *P. staleyi* (group D). Group C comprised '*R. apulia*' SM50 together with six OTU representatives.

Group A represents a taxon related to the genus *Rhodopirellula*, but so far no strain has been brought into culture. In this group, phylogenetic analysis of 28 clones with pairwise identities of 74–91% and 43 pyrosequencing OTUs suggest the presence of 60 novel species. Group A had 73–80% similarity to cultured *Rhodopirellula* strains, 72–77% to '*R. apulia*' SM50 and 67–73% to *B. marina* and *P. staleyi*.

Group D comprises sequences far away from *Rhodopirellula*. It includes OTUs with a relationship of equal distance to *Blastopirellula*, *Pirellula* and *Rhodopirellula*, with similarities of 64–73%. Group D formed in the tree (Fig. 3) a monophyletic branch with *Blastopirellula* and *Pirellula*.

DISCUSSION

The diversity of *Rhodopirellula* in coastal sediments of Sylt was characterized by 16S rRNA gene libraries, and by libraries and 454 sequencing of *carB* gene, with primers developed to target specifically the genus *Rhodopirellula*. Sylt was chosen for the diversity study as *Planctomycetes* were reported to be very abundant in the upper layers of sandy intertidal sediments on the island, accounting for between 3 and 19% of all cells (Musat *et al.* 2006).

For a planctomycete-enriched 16S rRNA gene study, the PLA886 probe developed for *in situ* hybridization (Neef *et al.* 1998) was used as a *Planctomycetes*-specific reverse primer for the generation of 16S rRNA gene libraries (Pynaert *et al.* 2003). This probe covered 91.6% of *Planctomycetaceae* and 91.5% of *Rhodopirellula* sequences of the SILVA database (Quast *et al.* 2013) and was selected because earlier *in situ* hybridization studies on the coastal sand under study were performed with this probe (Musat *et al.* 2006). As the *Planctomycetes*-specific forward primers Pla40F and Pla46F had low percentage matches within the target group (Pollet, Tadonléké and Humbert 2011), we used the general bacterial primer 8–27F as forward primer (Juretschko *et al.* 1998). The libraries yielded 119 *Planctomycetes* sequences of which 14 clustered more closely to *Rhodopirellula* then to other validly described *Planctomycetes*. Although ~12% of the sequences clustered closely to *Rhodopirellula*, we did not detect any of the cultured *Rhodopirellula* strains. Similar sequences were also found in water samples taken at Helgoland in the German Bight (Pizzetti *et al.* 2011). In that study, the *Rhodopirellula* related group accounted also for ~12% of the planctomycetal diversity. In a 16S rRNA V6 region 454 pyrosequencing study of Sylt sediments (project ICM_FIS_Bv6, vamps.mbl.edu, Huse *et al.* 2014), 4566 planctomycetal reads contained 86 reads affiliating to *R. baltica* SH1^T (1.8%) and 75 reads affiliating to strain SM50 (1.6%) (>95% identity over 60 bp, the V6 regions of SM50 and *R. baltica* had an identity of just below 95%). This deep sequencing study also indicated that cells of *Rhodopirellula* present a small fraction of all planctomycetes in Sylt sediments.

To get a better insight into the genus *Rhodopirellula*, we wanted to go beyond the resolution of the 16S rRNA gene and therefore studied the diversity of *carB* gene. Functional genes are less conserved than 16S rRNA genes and more suitable for a characterization at a higher taxonomic level. However, the dissimilarities between sequences of species within one genus and of species of closely related genera are too similar, so a reliable prediction of genus borders is not possible (Kim *et al.* 2014). The availability of nine genomes of *Rhodopirellula* strains allowed the correlation of the *carB* gene similarity with the ANI which showed that threshold value for the *carB* gene of 95% is well suited to separate closely related species, but the genus threshold could not be established. In these kinds of investigations, the threshold for species borders should be clearly outside the technical uncertainty of the sequencing technology. For example, the *rpoB* gene sequence has a higher degree of conservation with a species border threshold of 98.2% (Bondoso, Harder and Lage 2013), clearly above the ANI of shared genes between species making it less suitable for a diversity study.

The *carB* study revealed the presence of sequences with high similarity with *R. baltica* SH1^T, '*R. maiorica*' SM1, *R. rosea*, '*R. europaea*', '*R. islandica*' K833, *Rhodopirellula* sp. CS14 and six so far uncultured *Rhodopirellula* species within the group B, plus 66 novel species closely related to *Rhodopirellula* within the groups A and C. We also detected 50 new species of *Planctomycetes* in the group D which are not closely related to *Rhodopirellula*.

This study demonstrated a high diversity of *Rhodopirellula* in North Sea sediments from Sylt. The detection of sequences related to strains isolated from the Baltic Sea (*R. baltica*), Iceland ('*R. islandica*' K833) as well as from the Belgium coast and the Mediterranean Sea ('*R. europaea*') raises the question of the biogeography of these species. The dispersal of organisms with water currents may add to the diversity in Sylt sediments. This place is considered to be in contact with the water masses of the coastal current that originate from the North Atlantic Drift entering the North Sea at Scotland and from Atlantic waters entering through the English Channel (Otto *et al.* 1990).

In summary, the high taxonomic resolution of the *carB* gene amplicon, together with a calibration of thresholds derived

Table 1. List of strains used in the *carB* study and pairwise correlation of 16S rRNA and *carB* gene similarities and ANI to *R. baltica* SH1^T.

Strain	*carB* (%)	*16S rRNA* (%)	ANI (%)	*carB* acc number	Genome acc number
**Rhodopirellula baltica* SH1 DSM 10527 (Schlesner *et al.* 2004)	100	100	100	NC_005027	NC_005027
**Rhodopirellula baltica* WH47 DSM 24081 (Winkelmann and Harder 2009)	99.1	99.9	97.2	AFAR01000279	AFAR00000000
**Rbhodopirellula baltica* SH28 DSM 24038 (Schlesner *et al.* 2004)	97.7	99.9	96.8	AMCW01000040	AMCW00000000
**Rhodopirellula baltica* SWK14 DSM 24080 (Winkelmann and Harder 2009)	97.6	100	97	AMWG00000000	AMWG00000000
**'Rhodopirellula europaea'* 6C DSM 24037 (Winkelmann and Harder 2009)	93.1	99.9	88.1	ANMO01000116	ANMO00000000
**'Rhodopirellula europaea'* SH398 DSM 24039 (Schlesner *et al.* 2004)	92.7	99.9	88.2	ANOF01000144	ANOF00000000
**'Rhodopirellula sallentina'* SM41 DSM 24067 (Winkelmann and Harder 2009)	80.2	97.9	70.4	ANOH01000435	ANOH00000000
**'Rhodopirellula maiorica'* SM1 DSM 24050 (Winkelmann and Harder 2009)	77.7	96.2	77.7	ANOG01000438	ANOG00000000
**Rhodopirellula rubra* SWK7 DSM 24063 (Winkelmann and Harder 2009)	83.2	98.6	70.2	ANOQ01000052	ANOQ00000000
**'Rhodopirellula islandica'* K833 DSM 24040 (Winkelmann and Harder 2009)	87.8	99.5	81.1	LECT00000000	LECT00000000
Rhodopirellula rubra LF2 DSM 25459 (Bondoso *et al.* 2014)	82.2	97.5	–	KT284308	–
'Rhodopillula apulia' SM50 DSM 24084 (Winkelmann and Harder 2009)	75.3	93.7	–	KT284309	–
Rhodopirellula lusitana UC17 DSM 25457 (Bondoso *et al.* 2014)	79.3	96.3	–	KT284310	–
Rhodopirellula sp. CS14 DSM 24082 (Winkelmann and Harder 2009)	79.1	96.3	–	KT284306	–
Rhodopirellula rosea LHWP3 JCM 17759 (Roh *et al.* 2013)	78.8	94.7	–	KT284307	–
**Blastopirellula marina* DSM 3645 (Schlesner *et al.* 2004)	71.9	88.1	65.9	AANZ01000007	AANZ00000000
**Pirellula staleyi* DSM 6068 (Schlesner and Hirsch 1987)	73.3	83.9	65.2	NC_013720	NC_013720
Rubinisphaera brasiliensis DSM 5305 (Schlesner 1989)	63.5	83.9	–	NC_015174	–
**Gimesia maris* DSM 8797 (Bauld and Staley 1980)	61.9	86.7	64.1	ABCE01000003	ABCE01000003
Planctopirus limnophilus DSM 3776 (Hirsch and Müller 1986)	62.6	81.5	–	NC_014148	–
Singulisphaera acidiphila DSM 18658 (Kulichevskaya *et al.* 2008)	62.4	78.2	–	NC_019892	–
Isosphaera pallida ATCC 43644 (Giovannoni, Schabtach and Castenholtz 1987)	62.6	77.5	–	NC_014962	–
Maricaulis maris MCS10 (Abraham *et al.* 1999)	61.2	74.6	–	NC_008347	–
Deferribacter desulfuricans DSM 14783 (Takai *et al.* 2003)	53.6	72.7	–	NC_013939	–
Kuenenia stuttgartiensis (Schmid *et al.* 2000)	55.7	76.2	–	CT573071	–

*Strains used to calculate ANI and pairwise correlation to *carB* similarities in Fig. 2.

from a set of reference genomes in the genus of interest, provided a deep insight into the microdiversity of a genus, here *Rhodopirellula*, in the environment. The *carB* gene has more discriminatory power than the 16S rRNA gene when analyzing closely related *Rhodopirellula* species and it is suitable to discriminate strains on the intraspecies level and may be used for the taxonomic evaluation of the new isolates.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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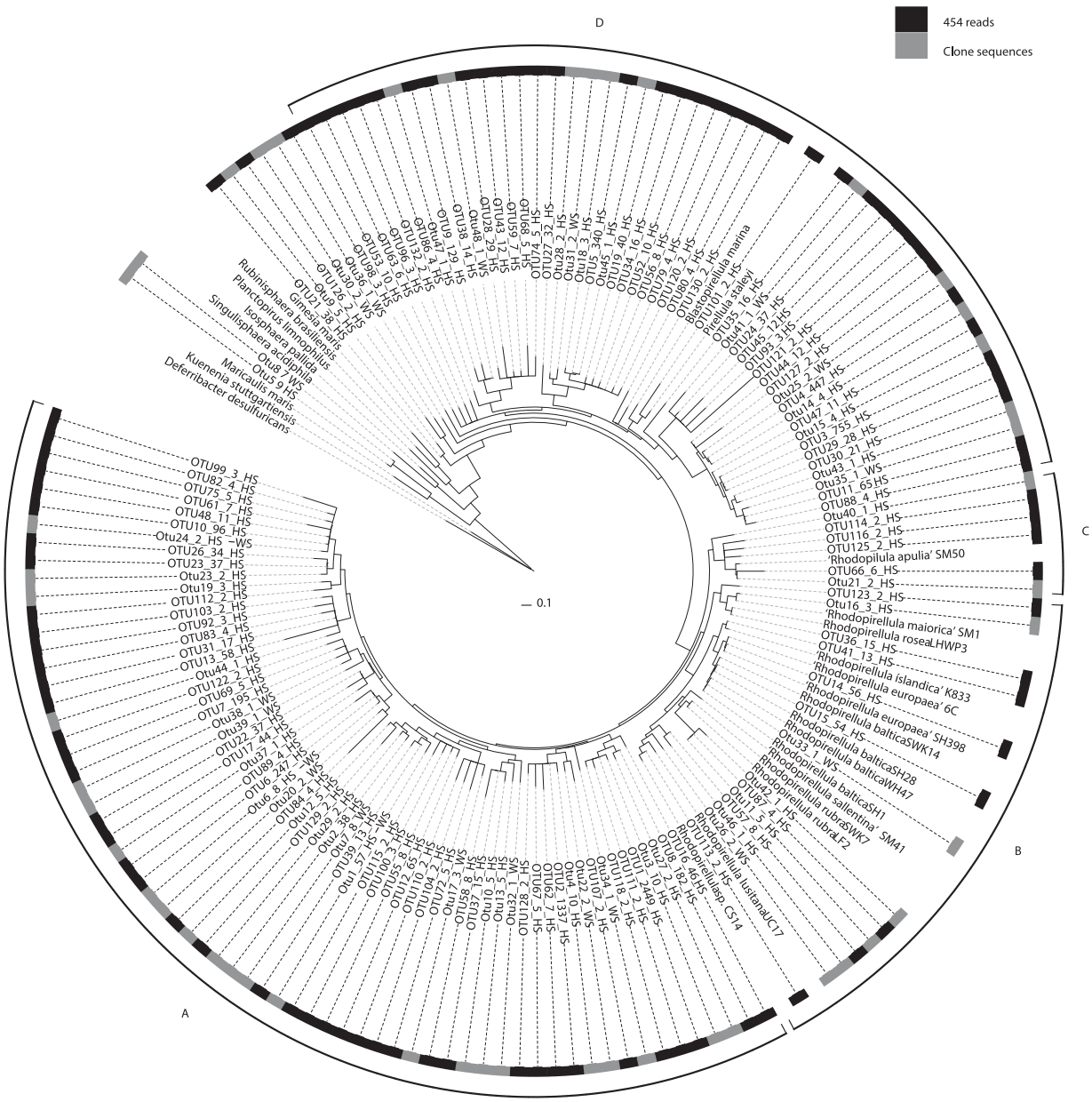

Figure 3. Phylogenetic ML tree of *carB* gene showing the affiliation of clone library OTU representative sequences clustered at 95% similarity, pyrosequencing OTU representative sequences clustered at 97% similarity and cultured *Planctomycetes* strains. Labeling: clone OTU representatives, Otu (number of OTU_number of sequences_sample site); pyrosequencing OTU representatives, OTU (number of OTU_number of reads_sample site). *Deferribacter desulfuricans* and *K. stuttgartiensis* were used as outgroup. Scale bar represents 0.1 substitutions per 100 nucleotides.

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