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

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Running title: *carB* diversity detects *Rhodopirellula* species

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

1. Introduction

The genus *Rhodopirellula* belongs to the widespread bacterial phylum *Planctomycetes* in the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) super-phylum (Wagner and Horn, 2006). The planctomycetes possess phenotypic characteristics unusual for the domain *Bacteria*, including reproduction by budding, and an intracellular membrane-bounded compartmentalization which extent 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 (van Teeseling *et al.* 2015, Jeske *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 like prawns and sponges (Fuerst *et al.*, 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, 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 *R. baltica* SH1^{T}. In a multilocus sequence analysis (MLSA) and in combination with DNA–DNA hybridization experiments, those isolates were arranged into 13 operational taxonomic units 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

68 definition can never be based solely on sequence similarity of 16S rRNA genes. Two
69 organisms with almost identical 16S rRNA gene sequences can still be recognized as
70 two different species based on DNA-DNA hybridisation (DDH) (Fox, Wisotzkey and
71 Jurtshuk, 1992). Stackebrandt and Ebers (2006) recommended that above a value
72 between 98.7 and 99 % in the 16S rRNA gene sequence similarity DDH would be
73 necessary. With the advent of rapid genome sequencing, DDH is being replaced by
74 determination of the average nucleotide identity (ANI) of shared genes between two
75 strains (Richter and Rosselló-Móra, 2009). Konstantinidis and Tiedje (2005) showed
76 that ANI values of 94–95 % correspond to 70% similarity in DNA-DNA
77 hybridization experiments, the current standard of the species definition. Besides the
78 16S rRNA gene, other molecular markers can be used to resolve bacterial
79 phylogenetic relationships. Recently, the *rpoB* gene, coding for the beta subunit of the
80 RNA polymerase, has been used for the taxonomic affiliation of *Planctomycetes*
81 strains (Bondoso, Harder and Lage, 2013).
82 The aim of this study was to investigate the diversity of *Rhodopirellula* in sandy
83 intertidal sediments of Sylt island, Germany, using as molecular markers 16S rRNA
84 and *carB* genes, the latter coding for the large subunit of carbamoylphosphate
85 synthetase. The *carB* gene was selected based on a comparison of genomes of nine
86 *Rhodopirellula* strains (Glöckner *et al.*, 2003; Klindworth *et al.*, 2014; Richter *et al.*,
87 2014a, 2014b; Richter-Heitmann *et al.*, 2014; Wegner *et al.*, 2014) and six related
88 planctomycetes following the requirements for candidate genes used in a species
89 prediction (Stackebrandt *et al.*, 2002).

90 **2. Materials and methods**

91

92 2.1 Sampling and DNA extraction
93 Samples from the upper 2 cm of sandy coarse sediment were obtained from two
94 locations, Hausstrand beach (55.01518 N, 8.43814 E) and Weststrand beach
95 (55,03840 N 8,38490 E) on the coast of Sylt island, Germany. Samples were collected
96 into 15 ml falcon tubes and frozen at -80°C till further processing. Genomic DNA
97 was extracted from 500 mg of sediment using FastDNA® Spin Kit for Soil (MP
98 Biomedicals, USA) according to manufacturer instructions. The quantity and quality
99 of extracted DNA was determined with a NanoDrop 100 Spectrophotometer (Thermo

100 Fisher Scientific, USA) followed by agarose gel electrophoresis and ethidium
101 bromide (EtBr) staining.
102
103 2.2 PCR amplification of 16S rRNA gene
104 The general bacterial 16S rRNA gene forward primer 8-27F (Juretschko *et al.*, 1998)
105 and the planctomycete-specific reverse primer PLA886 (Neef *et al.*, 1998) were used
106 to amplify partial 16S rRNA gene in a 30µl of PCR mixture containing 10-15 ng of
107 genomic DNA, 3.3 µM of each primer and 15 µl of GoTaq® Hot Start Colorless
108 Master Mix (Promega, USA). The template DNA was denatured for 4 min at 94°C,
109 followed by 30 cycles of 1 min at 94°C, 1 min at 65°C, 3 min at 72°C, and a final
110 extension of 10 min at 72 °C.
111
112 2.3 Design and PCR amplification of *carB* gene
113 Alignments of functional genes that were present in single copy in nine
114 *Rhodopirellula* and six other planctomycetes genomes (*Blastopirellula marina*,
115 *Gemmata obscuriglobus*, *Planctomyces maris*, *Planctomyces limnophilus*,
116 *Planctomyces staleyi* and *Kuenenia stuttgartiensis*) were used to identify suitable
117 marker genes. Criteria for marker gene regions included a unique PFAM model, an
118 annotated function, two conserved regions flanking 500-800 bases of high variability,
119 and primer sites conserved in all nine *Rhodopirellula* genome and very different in the
120 other planctomycetal genomes. Genes were selected after manual alignment
121 inspection of 373 candidate genes and primers were designed using the conservation
122 plots. For the *carB* gene, a forward degenerated primer at the position 2095-2114 (5´-
123 GCHCGBAACATGGAMGAAGC-3´) and a reverse degenerated primer at the
124 postion 2827-2808 (5´-CVGCGAKTTGGCTYTTKGCR-3´) were highly specific for
125 *Rhodopirellula* strains and used to generate 693bp long *carB* amplicons. The PCR
126 mixture contained 10-15 ng of genomic DNA, 3.3 µM of each *carB* primer, and a 2x
127 PCR master mix (Promega, USA) in 25 µl volume. The template DNA was denatured
128 for 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 60°C, 3 min at
129 72°C, and a final extension of 10 min at 72 °C.
130
131 2.4 Gene library construction and sequencing of 16S rRNA gene and *carB* amplicons
132 16S rRNA gene and *carB* PCR amplicons were purified on Sephadex™ G-50
133 Superfine columns (Amersham Biosciences AB, Uppsala, Sweden) and ligated into

134 the vector pCR4 applying the TOPO® TA cloning (Invitrogen™, USA). Inserts were
135 sequenced using the BigDye Terminator v3.1 Cyle Sequencing Kit (Applied
136 Biosystems, Carlsbad, CA, USA) on an ABI PRISM 3130xl Genetic Analyzer.
137
138 2.5 454 pyrosequencing of *carB* amplicon
139 In addition to the clone library, a *car*B amplicon from a Hausstrand sample was
140 analyzed by 454 pyrosequencing. PCR amplification was performed in two steps.
141 First PCR amplicons were obtained according to the aforementioned protocol with 20
142 cycles of amplification. The PCR products were purified with the QIAquick ® PCR
143 purification kit (Qiagen, Hilden, Germany) and 1 µl of purified PCR amplicon was
144 used for a second PCR amplification with fusion primers according to the
145 aforementioned protocol for 20 cycles. Fusion primers contained in front of the *carB*
146 primers a linker required for 454 sequencing; forward fusion primer (5´-
147 GATGGCCATTACGGCC - GCHCGBAACATGGAMGAAGC-3´), reverse fusion
148 primer (5´- GGTGGCCGAGGCGGCCACACGT -
149 CVGCGAKTTGGCTYTTKGCR-3´). The amplicon was purified and sequenced on a
150 454 GS FLX sequencer at the Max Planck Genome Centre, Cologne.
151
152 2.6 Analysis of 16S rRNA gene and *carB* clone library sequences
153 16S rRNA gene and *carB* sequences were analyzed with Sequencing Analysis 5.2
154 (Applied Biosystems, Carlsbad, CA, USA) and truncated within Sequencer 4.6 (Gene
155 Codes, Michigan, USA). The 16S rRNA gene sequences were aligned in ARB
156 software (Ludwig *et al.*, 2004) using the SINA_aligner (Pruesse, Peplies and
157 Glöckner, 2012). Manual refinement was conducted by comparison with their closest
158 relatives. The *carB* sequences were first translated into protein sequences and visually
159 inspected for the presence of stop codons. Translated sequences were aligned with
160 MAFFT (Katoh, Asimenos and Toh, 2009) and the nucleotide sequence alignment
161 was generated according to the protein alignment. Distance matrices of 16S rRNA
162 gene and *carB* sequences were calculated using the neighbor-joining method as
163 implemented in ARB. OTU clustering was performed based on those matrices using
164 the software Mothur v 1.29.1 (Schloss *et al.*, 2009). Representative OTU sequences
165 were used to calculate phylogenetic maximum likelihood (ML) trees in ARB using
166 RAxML 7.0.4 (Stamatakis, 2006) with 50% minimal similarity filter.
167

168 2.7 Analysis of *carB* pyrosequencing reads

169 Pyrosequencing reads were first processed in Mothur. The analysis included removal
170 of primer sequences, quality control to remove sequences with ambiguous
171 nucleotides, denoising and removal of chimeric sequences using the UCHIME
172 algorithm (Edgar *et al.*, 2011). Sequences were translated, inspected for stop codons
173 and aligned with MAFFT. The corresponding nucleotide sequence alignment was
174 generated according to the protein alignment. A distance matrix was calculated using
175 the neighbor-joining method as implemented in ARB and OTU clustering was
176 performed based on that matrix using the software Mothur. OTU representatives of
177 454 reads were aligned together with the OTU representative sequences of the *carB*
178 clone libraries and the alignment confidence scores were assessed using the
179 GUIDANCE algorithm (Penn *et al.*, 2010b) implemented in the GUIDANCE web
180 server (Penn *et al.*, 2010a). Sequences below the confidence score of 0.826 were
181 removed from the analysis. The 454 reads were then placed in the reference ML *carB*
182 tree using the evolutionary placement algorithm (EPA) (Berger, Krompass and
183 Stamatakis, 2011) implemented in RAxML 8 (Stamatakis, 2014).

184

185 2.8 Accession numbers

186 The 16S rRNA gene and *carB* nucleotide sequences obtained in this study were
187 deposited in the Genbank Acc (Accession numbers will be provided in the later stage)

188

189 **3. Results**

190 **3.1. Analysis of 16S rRNA gene clone library**

191 The clone libraries from both sample sites yielded 158 sequences, of which 119
192 affiliated with *Planctomycetes*. The 119 planctomycetal sequences were aligned
193 covering the 16S rRNA gene positions 57-906 (numbering related to *E. coli* sequence)
194 and grouped into OTUs using either a genus-related threshold (95%), resulting in 63
195 OTUs, or a species-related threshold (97%), resulting in 73 OTUs. The phylogenetic
196 analysis placed 7 OTUs at the 97% threshold closer to the genus *Rhodopirellula* than
197 to its next validly described relative *Blastopirellula*, but none of the clone sequences
198 affiliated closely with the species in the genus *Rhodopirellula* (Fig 1). OTUs with the
199 closest affiliation to *Rhodopirellula* formed a group with the strain SM50, which

200 represents a novel genus based on a gene identity of 93.7% for the complete 16S
201 rRNA gene (Winkelmann *et al.*, 2010) and is tentatively named `*Rhodopilula apulia*`.
202

203 **3.2. Correlation between *carB* gene and ANI**

204 To resolve intra and interspecies relationship and to define the species threshold for
205 the partial *carB* gene (693bp) used in this study, the pairwise similarity values of the
206 *carB* gene were plotted against the ANI values between each pair of genomes (Fig.2).
207 The ANIs were determined by using the *in silico* DNA-DNA hybridization method of
208 the JSpecies software (Richter and Rosselló-Móra 2009). Strains used for the
209 calculation and *carB* identities of strains to *Rhodopirellula baltica* SH1^T are shown in
210 Table 1. Genome analyses of four strains of *R. baltica* and two strains of the related
211 species `*Rhodopirellula europaea*` established an intraspecies identity of the amplicon
212 region of at least 97.4%. The pairwise interspecies average nucleotide identity (ANI)
213 for the two species was 88% for the common genes in the genomes and less than
214 93.1% for the *carB* amplicon. Thus, a threshold of 95% was well suited to separate
215 closely related *Rhodopirellula* species. *B. marina* and *P. staleyi* had higher sequence
216 similarity (81.1%) than some strains within *Rhodopirellula* e.g. `*R. sallentina*` SM41
217 and `*R. maiorica*` SM1 (77.7%), so a genus threshold could not be established solely
218 on the basis of *carB* partial gene sequences.
219 The *carB* sequences of the 454 pyrosequences covered an alignment of 204 base in
220 length. In contrast to the 693 base alignment of Sanger sequences, this *carB* region
221 was slightly higher conserved, resulting in a species border threshold of 97%.
222

223 **3.3 Analysis of *carB* sequences**

224 Two *carB* clone libraries gave 233 sequences which clustered into 48 OTUs on a 95%
225 similarity threshold. The representative sequences of the OTUs and the *carB*
226 sequences of *Planctomycetes* stains were used for the phylogenetic analysis (Fig 3).
227 Strains and corresponding *carB* gene accession numbers are listed in Table 1. The tree
228 revealed that the majority of the OTUs were more closely related to *Rhodopirellula*
229 than to *Blastopirellula*. We detected one OTU that affiliated with *R. baltica* SH1^T
230 with 99.6% similarity and one OTU that clustered with `*R. maiorica*` SM1 with
231 96.7% similarity.
232 A deeper insight into the species diversity was obtained by 454 pyrosequencing of a
233 *carB* amplicon. After preprocessing, the dataset included 7763 reads with a length of

234 204 bp. The *carB* sequences of the genomes had indicated for the amplicon size of
235 204 bp a higher species border threshold of 97%. Applying this threshold, the reads
236 clustered in 290 OTUs, of which 157 OTUs were singletons. Chao1, a conservative
237 richness estimator, predicted the presence of 650 OTUs (548-792). After removing
238 singletons and sequences with alignment confidence score below 0.826, 95 OTUs
239 remained and were added to an already constructed ML tree of *carB* clone and strain
240 sequences (Fig 3). Considering the relatedness to the *Rhodopirellula* strain sequences,
241 *carB* sequences obtained in this study clustered in 4 groups.
242 Group B contained cultured *Rhodopirellula* strains, clone library sequences of *R.*
243 *baltica* SH1^{T} and `*R. maiorica*` SM1 as well as pyrosequencing reads of *R. baltica*, *R.*
244 *rosea*, `*R. europaea*`, *Rhodopirellula* sp. CS14 and ´*R. islandica*´ K833. In absolute
245 numbers, group B included 154 reads and 13 clone sequences. In addition to known
246 strains, phylogenetic analysis of the group B suggested a presence of six so far
247 uncultured *Rhodopirellula* species. Pairwise identities within group B were 73-100%,
248 between members of the group to ´*R. apulia*´ SM50 (group C) 72-75% and to *B.*
249 *marina* and *P. staleyi* (group D) 67-73% and 67-73%, respectively. Group C
250 comprised ´*R. apulia*´ SM50 together with six OTU representatives.
251 Group A represents a taxon related to the genus *Rhodopirellula*, but so far no strain
252 has been brought into culture. In this group, phylogenetic analysis of 28 clones with
253 pairwise identities of 74-91% and 43 pyrosequencing OTUs suggest the presence of
254 60 novel species. Group A had 73-80% similarity to cultured *Rhodopirellula* strains,
255 72-77% to ´*R. apulia*´ SM50 and 67-73% to *B. marina* and *P. staleyi*.
256 Group D comprises sequences far away from *Rhodopirellula*. It includes OTUs with a
257 relationship of equal distance to *Blastopirellula*, *Pirellula* and *Rhodopirellula*, with
258 similarities of 64-73%. Group D formed in the tree (Fig. 3) a monophyletic branch
259 with *Blastopirellula* and *Pirellula*.
260

261 **4. Discussion**

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

268 For a planctomycete-enriched 16S rRNA gene study, the PLA886 probe developed
269 for *in situ* hybridization (Neef *et al.*, 1998) was used as a *Planctomycetes*-specific
270 reverse primer for the generation of 16S rRNA gene libraries (Pynaert *et al.*, 2003).
271 This probe covered 91.6% of *Planctomycetaceae* and 91.5% of *Rhodopirellula*
272 sequences of the SILVA database (Quast *et al.*, 2013) and was selected because
273 earlier *in situ* hybridization studies on the coastal sand under study were performed
274 with this probe (Musat *et al.*, 2006). As the *Planctomycetes* specific forward primers
275 Pla40F and Pla46F had low percentage matches within the target group (Pollet,
276 Tadonléké and Humbert, 2011), we used the general bacterial primer 8-27F as
277 forward primer (Juretschko *et al.*, 1998). The libraries yielded 119 *Planctomycetes*
278 sequences of which 14 clustered more closely to *Rhodopirellula* then to other validly
279 described *Planctomycetes*. Although ~12% of the sequences clustered closely to
280 *Rhodopirellula,* we did not detect any of the cultured *Rhodopirellula* strains. Similar
281 sequences were also found in water samples taken at Helgoland in the German Bight
282 (Pizzetti *et al.*, 2011). In that study, the *Rhodopirellula* related group accounted also
283 for ~12% of the planctomycetal diversity. In a 16S rRNA V6 region 454
284 pyrosequencing study of Sylt sediments (project ICM_FIS_Bv6, vamps.mbl.edu,
285 Huse *et al.*, 2014), 4566 planctomycetal reads contained 86 reads affiliating to
286 *Rhodopirellula baltica* SH1^{T} and 75 reads affiliating to strain SM50 (more than 95%
287 identity over 60 bp). The V6 regions of SM50 and *R. baltica* had an identity of just
288 below 95%, of *B. marina* and *R. baltica* had an identity of 83%. This deep sequencing
289 study also indicated that cells of *Rhodopirellula* present a small fraction of all
290 planctomycetes in Sylt sediments.
291 To get a better insight into the genus *Rhodopirellula*, we wanted to go beyond the
292 resolution of the 16S rRNA gene and therefore studied the diversity of *carB* gene.
293 Functional genes are less conserved than 16S rRNA genes and more suitable for a
294 characterization at a higher taxonomic level. However, the dissimilarities between
295 sequences of species within one genus and of species of closely related genera are too
296 similar, so a reliable prediction of genus borders is not possible (Kim *et al.*, 2014).
297 The availability of nine genomes of *Rhodopirellula* strains allowed the correlation of
298 the *carB* gene similarity with the ANI which showed that threshold value for the *carB*
299 gene of 95% is well suited to separate closely related species, but the genus threshold
300 could not be established. In these kinds of investigations, the threshold for species
301 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 British Channel (Otto *et al.*, 1990).

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

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336

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