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"*Candidatus* Haloectosymbiotes riaformosensis" (*Halobacteriaceae*), an archaeal ectosymbiont of the hypersaline ciliate *Platynematum salinarum*☆

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ABSTRACT

The novel ciliate *Platynematum salinarum* (*Scuticociliatia*) was isolated only recently from a thalassohaline solar saltern pond (12%) in Portugal. Scanning electron microscopy showed numerous bacterial-shaped cells covering the complete surface of the ciliate. The rod-shaped epibionts were identified and characterized following the "Full-Cycle rRNA Approach". The almost full-length 16S rRNA gene sequence was obtained using archaeal-specific primers and two species-specific probes were designed for fluorescence *in situ* hybridization. The 16S rRNA gene sequence of the epibiotic cells showed 87% sequence identity with the type strain sequence of the closest characterized species *Halolamina pelagica*. Phylogenetic reconstructions affiliated the novel organism to the genus *Halolamina* (*Halobacteria*, *Archaea*). Attempts to isolate the epibionts failed and, therefore, growth experiments incorporating the antibiotic anisomycin were conducted in order to investigate the potential symbiotic relationship between *P. salinarum* and the epibionts. The results suggested an obligate symbiosis between the two organisms and revealed the first symbiotic representative of the *Halobacteria*. Based on the phylogenetic analyses and growth experiments we propose the classification of this novel organism in a new genus, with the taxon name "*Candidatus* Haloectosymbiotes riaformosensis".

Introduction

Novel prokaryotes, for which characteristics required for formal description according to the International Code of Nomenclature of *Bacteria* are lacking, can be given a provisional *Candidatus* status [35]. Predominantly, this applies to prokaryotes from which it is difficult (or not possible) to obtain pure cultures, which is a prerequisite for the identification of specific characteristics needed for a formal description. This is a common phenomenon encountered, for example, in prokaryote symbionts of ciliates [6,15]. To assign a provisional *Candidatus* status, the following informations are required: (i) relatedness to the nearest neighbor based on, for example, a taxonomic marker sequence; (ii) an assay such as *in situ* probing to verify authenticity, and (iii) phenotypic properties that can be used as a starting point for further investigation [35].

Members of the family *Halobacteriaceae* (*Archaea*) are obligate extreme halophiles thriving in a great variety of hypersaline environments (*e.g.* [19,31,47,52,53]). The minimal requirements for growth of *Halobacteriaceae* include salt concentrations above 1.5 M NaCl, with optimal growth between 2.0 and 4.5 M NaCl [24,37]. The members of this group have adapted their metabolism and protein amino acid compositions to environmentally high concentrations of salts, given that they accumulate internal cation concentrations as compatible solutes that may reach 5 M of potassium [38]. *Halobacteria* are the key players in high saline environments and generally represent the dominant prokaryotic communities. The family *Halobacteriaceae* was established in 1974 [21] with the two genera *Halococcus* and *Halobacterium*. Since then, the diversity of described taxa in this family has expanded tremendously, and comprised 36 genera and 129 species when reviewed in 2012 [40]. Besides high salt concentrations as a growth requirement, common traits shared by all *Halobacteriaceae* include the lack of a peptidoglycan cell wall, structural stability, and unusual

☆ *Note*: Nucleotide sequence data reported is available in the GenBank database under the accession number KF736828.

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pigmentation [30]. Furthermore, all species described to date are free-living.

The current study presents the first record of a new *Halobacteriaceae* species as an ectosymbiont of a small hypersaline ciliate (*Platynematum salinarum*) isolated from the Ria Formosa solar salterns in Faro, Portugal. Scanning electron microscopy (SEM) revealed that a prokaryotic epibiont grew as a dense coat on the ciliate's membrane. Attempts to separate the epibiont from the scuticociliate failed. Therefore, a "Full-Cycle rRNA Approach" [1] was applied in order to identify the epibiont. Based on the information retrieved, a symbiotic relation with the ciliate is hypothesized. In addition, given the information obtained from the epibiotic archaeon, we propose a *Candidatus* status for it with the name "*Candidatus* Haloectosymbiotes riaformosensis", according to Murray and Schleifer [34].

Materials and methods

Sampling, cultivation and identification

The detected epibiont was growing on the membrane of the small scuticociliate *Platynematum salinarum* [18]. *P. salinarum* was isolated from a solar saltern pond with a salt concentration of 12% in the Ria Formosa natural park (37°01′ N, −7°96′ E), Faro, Portugal. It was cultivated in sterilized salt medium (ASW, "artificial sea water", Instant Ocean, Aquarium Systems, Ohio, USA) adjusted to 12% salinity with NaCl and amended with two to four wheat grains per 25 mL salt medium as a food source in order to support growth of indigenous bacteria.

Scanning electron microscopy (SEM)

SEM followed the protocol described by Kolodziej and Stoeck [27] but with small modifications. Briefly, 8 mL of *P. salinarum* culture were fixed for 2 h at 4 °C with 25% glutaraldehyde (final concentration 10% in 0.1 M cacodylate buffer), filtered onto Transwell polycarbonate membrane filters (24 mm, 3 μm, Corning Inc., New York, USA) and washed three times with cacodylate buffer. Filters were covered with 10–15 drops of osmium tetroxide in 0.1 M cacodylate buffer (final concentration 1%), incubated for 1 h and washed twice with cacodylate buffer. Afterwards, filters were taken through a dehydration series of 30, 50, 70, 80, 90, 95 and 100% of ethanol-cacodylate buffer mixtures for 10–15 min. Finally, filters were transferred to a 1:1 hexamethyldisilzane-ethanol mix and washed three times with hexamethyldisilizane for 15–20 min. Until the final washing step, filters were not allowed to dry out at any time. All incubations were at room temperature. Prepared filter pieces were then attached to a carbon adhesive tab and mounted on a SEM specimen holder. Mounted specimens were sputter coated with gold using a Cressington 108 auto sputter coater (Cressington Scientific Instruments Ltd., Watford, UK) and visualized with a Carl Zeiss SUPRA 55VP scanning electron microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and an accelerating voltage of 5 keV.

DNA extraction and 16S rRNA gene sequencing

Single specimen of *P. salinarum* carrying the ectosymbiont were isolated from a culture, washed three times in sterile salt medium and starved for 24 h in sterile salt medium in order to digest already ingested prokaryotes. Then, individual cells were picked, transferred into sterile PCR tubes containing the PCR reaction mix (see below) and subjected to single-cell PCR. Since it was unknown whether the ectosymbiont belonged to the domain *Bacteria* or *Archaea*, different PCR reactions targeting the gene coding for the small ribosome subunit (16S rRNA gene) for each domain were conducted. To amplify the archaeal 16S rRNA gene the archaea-specific forward primer 21F (5′-TTCCGGTTGATCCTGCCGGA-3′ [13]) and the universal reverse primer U1517 (5′-ACGGCTACCTTGTTACGACTT-3′ [51]) were used. For bacterial 16S rRNA gene amplification, the bacterial primer pair Bact8F (5′-AGAGTTTGATCMTGGCTC-3′ [23]) and U1517 was used. The PCR reaction mix included dNTPs (10 μmol each; Axon, Germany), 100 μmol μL^{-1} of a forward and a reverse primer (each with a final concentration of 0.5 μM), 0.5 μl HotStar Taq (5 U μL^{-1}, 2.5 U final concentration; Qiagen, Germany) and 5 μL of 10× Coral buffer (1× final concentration; Qiagen). The reaction mix was filled with PCR water to a final volume of 50 μL. The PCR program for amplification of the bacterial and archaeal 16S rRNA genes consisted of an initial 5 min denaturation at 95 °C, followed by 30 identical amplification cycles (denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 60 s), and final elongation at 72 °C for 10 min.

As PCR products were only obtained using the archaea-specific primer set, all following steps were conducted with the archaeal gene product. The PCR product was purified (MinElute PCR purification kit, Qiagen, Germany), ligated into pGEM-T vector (PGEM-T Vector Systems kit, Promega GmbH, Germany) and transformed into OneShot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Germany), according to the manufacturer's instructions. Plasmids were prepared from overnight cultures using the FastPlasmid Mini kit (5 Prime GmbH, Hamburg, Germany) and sequenced bidirectionally using M13 sequencing primers on an Applied Biosystems (ABI) 3730 XL DNA Stretch Sequencer with the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction kit.

Phylogenetic reconstructions

The forward and reverse sequences obtained were quality checked (including vector and primer trimming), PHRED and PHRAP analyzed, and assembled to one near-complete 16S rRNA gene sequence using the software CodonCodeAligner v.4.0.4 (CodonCode Corporation, Dedham, MA). Phylogenetic inference was carried out using the ARB software package [32]. The almost complete sequence was automatically aligned using SINA aligner [45] against LTP 111 database [55] reference alignments and manually inspected in order to correct inaccurately misplaced bases. The treeing approach was undertaken using three different algorithms: maximum likelihood using the program RAxML [49], and neighbor-joining and maximum parsimony as implemented in the ARB software package [32], using different datasets and filters (see the respective figure legends). The sequence accession number of the epibiont is KF736828.

Probe design

Two different 16S rRNA-targeted oligonucleotide probes specific for the detected epibiont were designed using NCBI's Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Default program parameters were applied except for the primer melting temperature (T_m Min = 50, T_m Opt = 55, T_m Max = 63). Suggested probes were reverse complemented and checked for GC-content, melting temperature and self-complementarity (hairpins or dimers) according to the guidelines of Hugenholtz et al. [26]. Probe specificities were checked against GenBank's nr nucleotide database and by hybridization assays with environmental samples (original samples of the environment from which the host ciliate was isolated). Stringency was adjusted by applying different formamide concentrations (0–40%) using the fluorescence *in situ* hybridization protocol described below.