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# Overlooked Diversity of Ultramicrobacterial Minorities at the Air-Sea Interface

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**Abstract:** Members of the Candidate phylum Patescibacteria, also called Candidate Phyla Radiation (CPR), are described as ultramicrobacteria with limited metabolic capacities. Wide diversity and relative abundances up to 80% in anaerobic habitats, e.g., in groundwater or sediments are characteristic for *Candidatus* Patescibacteria. However, only few studies exist for marine surface water. Here, we report the presence of 40 patescibacterial candidate clades at air-sea interfaces, including the upper water layer, floating foams and the sea-surface microlayer (SML), a < 1 mm layer at the boundary between ocean and atmosphere. Particle-associated (>3 µm) and free-living (3–0.2 µm) samples were obtained from the Jade Bay, North Sea, and 16S rRNA (gene) amplicons were analyzed. Although the abundance of *Cand.* Patescibacteria representatives were relatively low (<1.3%), members of *Cand.* Kaiserbacteria and *Cand.* Gracilibacteria were found in all samples. This suggests profound aerotolerant capacities of these phylogenetic lineages at the air-sea interface. The presence of ultramicrobacteria in the >3 µm fraction implies adhesion to bigger aggregates, potentially in anoxic niches, and a symbiotic lifestyle. Due to their small sizes, *Cand.* Patescibacteria likely become aerosolized to the atmosphere and dispersed to land with possible implications for affecting microbial communities and associated processes in these ecosystems.

**Keywords:** Candidate phyla radiation; bacteria; sea-surface microlayer; neuston; foam; 16S rRNA sequencing; aerosols; air-sea interface

## 1. Introduction

Ultra-small bacteria of the Candidate Phylum Patescibacteria [1] also called Candidate Phyla Radiation (CPR) comprise a high biodiversity within the bacterial domain [2,3]. *Cand.* Patescibacteria include Microgenomates (OP11), Parcubacteria (OD1), Dojkabacteria (WS6), Katanobacteria (WWE3), Gracilibacteria, Berkelbacteria (ACD58), Peregrinibacteria (PER), Kazania, ABY1, CPR2, and Saccharibacteria (TM7) [4]. Due to reduced cell and genome sizes only limited metabolic capacities are hitherto described for these bacteria [5]. Therefore, *Cand.* Patescibacteria have been suggested to be symbionts of other microbes [6–9] or even considered virus-like [10]. Because of the frequent lack of

a respiratory chain [5,10] and their presence in oxygen-deprived and anaerobic ecosystems [11–14] a fermentative-based lifestyle has been suggested for most *Cand.* Patescibacteria [10]. However, *Cand.* Patescibacteria were also detected in oceans at a depth of 5 m [15], aerobic surface water of a thermokarst lake ecosystem [16], deep oxic Lake Baikal [17], and oxic mixolimnion of Lake Parvin [18]. For their oxic lifestyle an unusual respiratory metabolism especially of *Cand.* Parcubacteria has been proposed [19,20]. In addition, recent work suggested that nano-sized prokaryotes including *Cand.* Patescibacteria could contribute to carbon cycling in the oceans [21] and supported by a high diversity of *Cand.* Patescibacteria, important implications for exchange processes across the air-sea boundary can be inferred [22].

Microorganisms populating the sea-surface microlayer (SML), a < 1 mm layer at the air-sea boundary, are collectively referred to as neuston and encompass cell numbers of $2 \times 10^{23}$ on Earth [23]. The biofilm-like, gelatinous nature of the SML [24] with its distinct physicochemical features compared to the underlying water (reviewed by Cunliffe, et al. [25]) and spontaneously emerging surface phenomena, such as slicks and foams found increasing research interest as bacterial habitats during the last decade [24,26–29]. However, very little is known about the role of ultramicrobacteria in this elusive ecotone between atmosphere and hydrosphere. In recent studies, *Cand.* Patescibacteria were detected in freshwater SML in Lake Parvin, France with slight enrichments over the epilimnion [30]. Furthermore, *Cand.* Patescibacteria were present in air samples collected over the Southern Ocean [31], but their origin remained unclear.

In this study, we hypothesize that also saltwater bacterial communities in 1 m deep water, the SML, and foam harbor ultramicrobacteria of the phylum *Cand.* Patescibacteria, and that these are potential components of aerosols. We differentiate the 0.2–3 µm ("free-living") and the >3 µm fraction ("particle-associated") in floating foams, the SML and the underlying water since *Cand.* Patescibacteria in the 0.2–3 µm fraction are rather objects for aerosolization due to their small size, while in the >3 µm fraction they are more likely to live symbiotic or to adhere to organic material.

## 2. Experiments

Cell count and 16S rRNA amplicon data for this study were extracted and re-analyzed from a previous study [27]. Samples of foam, SML and underlying, i.e., 1 m deep water were sampled in the Jade Bay, North Sea, offshore Wilhelmshaven, Germany (Figure 1). SML and floating foams (Figure 1) were collected using the glass plate method [32], and the glass plate rinsed with 70% ethanol. Underlying water from 1 m depth was sampled with a syringe connected to a weighted hose. For prokaryotic cell counts, samples were fixed with 1% final concentration of glutaraldehyde, incubated for 1 h at room temperature and frozen at −80 °C until further processing. Due to their sticky nature and particle content, foam samples were pre-filtered by gravity onto CellTrics® 50 µm mesh-size filters (Sysmex Partec, Muenster, Germany). Cells were stained with SYBR® Green I Nucleic Acid Gel Stain (9x in final concentration, Thermo Fisher Scientific, Darmstadt, Germany) and measured on a flow cytometer (C6 Flow-Cytometer, BD Bioscience, San Jose, CA, USA) following a protocol by Giebel, et al. [33]. Here, prokaryotes were gated/separated into high (HNA) and low (LNA) nucleic acid content cells according to Proctor, et al. [34], assuming that the LNA type contains small genomes [35] and thus includes *Cand.* Patescibacteria.

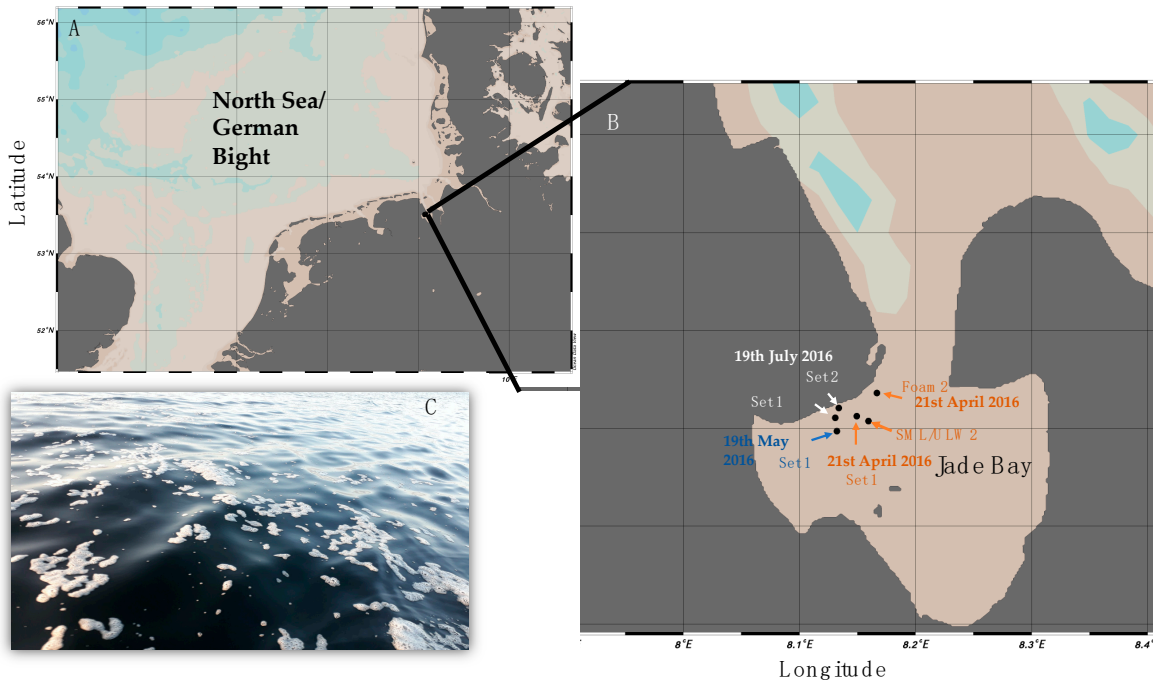


**Figure 1.** (**A**) Sampling stations in the Jade Bay, North Sea/German Bight, Germany; (**B**) A whole sampling set refers to foam/sea-surface microlayer (SML)/1-m depth = underlying water (ULW). As indicated, on one occasion foam and SML/ULW were collected in different places. Coordinates for Set 2 from 19 May 2016 are missing. (**C**) The occurrence of patchy sea foam floating at the sea surface (Photo: Janina Rahlff). Map was generated using Ocean Data View [36].

Samples were sequentially filtered onto 3 µm and 0.2 µm polycarbonate filter membranes (Merck Millipore, Darmstadt, Germany). Extraction of DNA and RNA were simultaneously performed using the DNA + RNA + Protein Extraction Kit (Roboklon, Berlin, Germany) with slight modifications from the manufacturer's protocol [37]. Remaining genomic DNA in RNA samples was digested on-column with 3 U of DNase, and RNA was used in a PCR to check for further contamination with DNA. Synthesis of cDNA was performed using the NG dART Kit (Roboklon, Berlin, Germany), 10 ng of RNA and the primer 1492R (5'-GGTTACCTTGTTACGACTT-3', [38]) in a run of 60 min. at 50 °C followed by 5 min. at 85 °C. Quantification of DNA and cDNA concentrations was done using the Quant-iT$^{TM}$ PicoGreen$^{TM}$ dsDNA assay (Thermo Fisher Scientific, Darmstadt, Germany). Amplicon and index PCR, subsequent quality checks, and sequencing of the bacterial 16S rRNA (gene) were conducted using Illumina MiSeq by a third-party service (Eurofins Genomics, Ebersberg, Germany). For amplicon PCR, 35 and 25 cycles were done for DNA and cDNA templates, respectively and by using the primer set Bakt_341F/805R [39].

Paired-end sequence reads were assembled using QIIME 1.9.1 [40] and evaluated using the SILVA NGS pipeline [41] including quality checks in compliance with SINA-based alignments [42], where PCR artifacts and non-SSU reads were excluded. Sequence reads were reanalyzed using SILVA SSU138 [4] as basis in ARB [43] and merging reads assigned to *Cand.* Patescibacteria without changing the global tree topology using the ARB parsimony tool. In the case of 98% sequence identity, classification of the ref sequence was mapped to all members of the respective cluster and to their replicates. The threshold for best BLAST hit acceptance was (sequence identity + alignment coverage)/2 ≥ 93%, and otherwise assigned to "unclassified". Sequence reads were deposited in the European Nucleotide Archive (ENA) under accession number PRJEB34343. Representative assembled reads assigned to *Cand.* Patescibacteria were deposited at GenBank under accession number MW167660-MW167765. The number of *Cand.* Patescibacteria related OTUs was compared between the three different habitats,

two nucleic acid types and two filtered fractions using a Kruskal–Wallis with a post hoc Tukey's HSD test to find for significant differences at the 95% significance level using PAST [44].

## 3. Results and Discussion

Several studies investigated the dominant bacterial community composition of the SML and foam [45–48]. However, potentially due to their low abundance, patescibacterial ultramicrobacteria have been neglected in marine neuston habitats to date. In this study, we use the name *Cand.* Patescibacteria [49] to cover all sequences within the "superphylum" Candidate Phyla Radiation (CPR) as classified in the 16S rRNA database SILVA SSU 138 [4,50]. This also includes the candidate groups Microgenomates (OP11), Parcubacteria (OD1), WS6, WWE3, Candidatus Berkelbacteria, Peregrinibacteria, and Saccharibacteria (TM7). The separation of >3 µm for particle-associated (PA) and 0.2–3 µm filtration for free-living (FL) bacteria is common practice in microbial ecology, although many ultra-small bacteria will pass 0.2 µm filter (reviewed by Nakai [51,52]). However, members of the TM7 are described to change their cellular morphology from very small cocci to large filaments and are thereby captured by >0.2 um filtration [53]. In our study, we found 15–369 different OTUs (Figure 2) of 40 different *Cand.* Patescibacteria clades across all samples in a relative abundance between 0–1.4% of the total community (Figure 3).

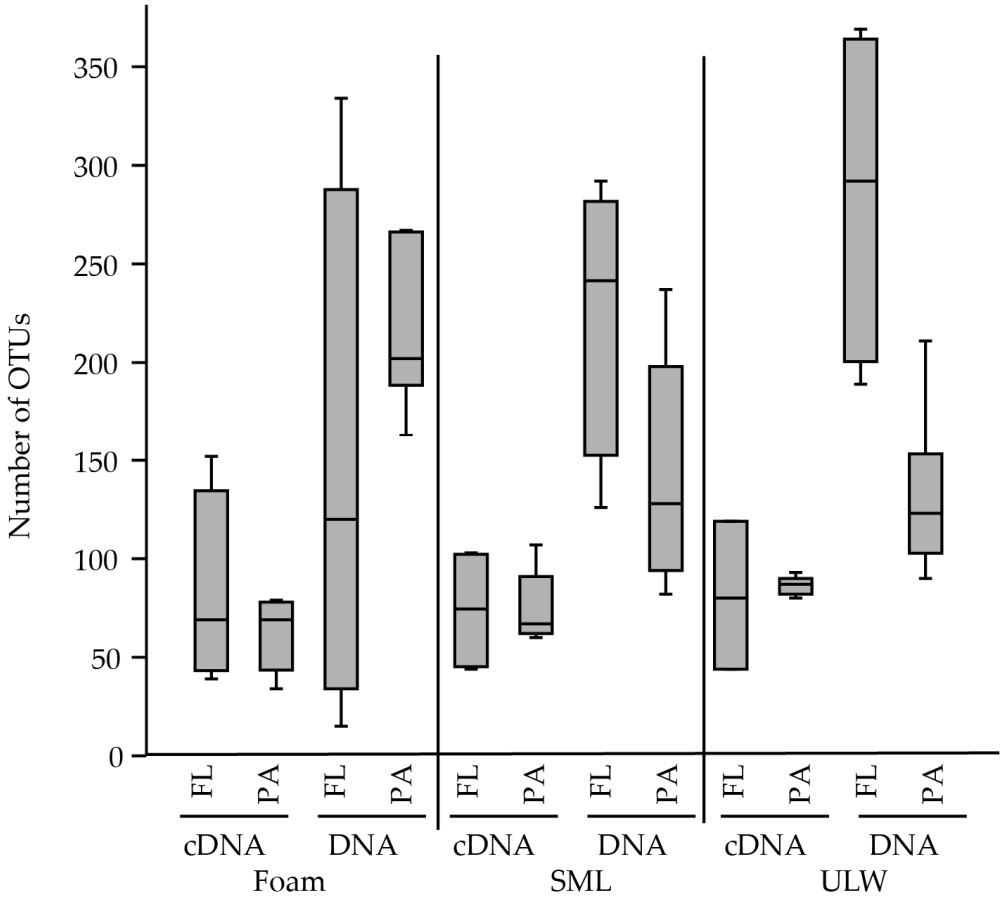


**Figure 2.** Median of patescibacterial OTUs per habitat, i.e., foam, sea-surface microlayer (SML) and underlying-water (ULW). Samples were further divided into free-living (FL) and particle-associated (PA).

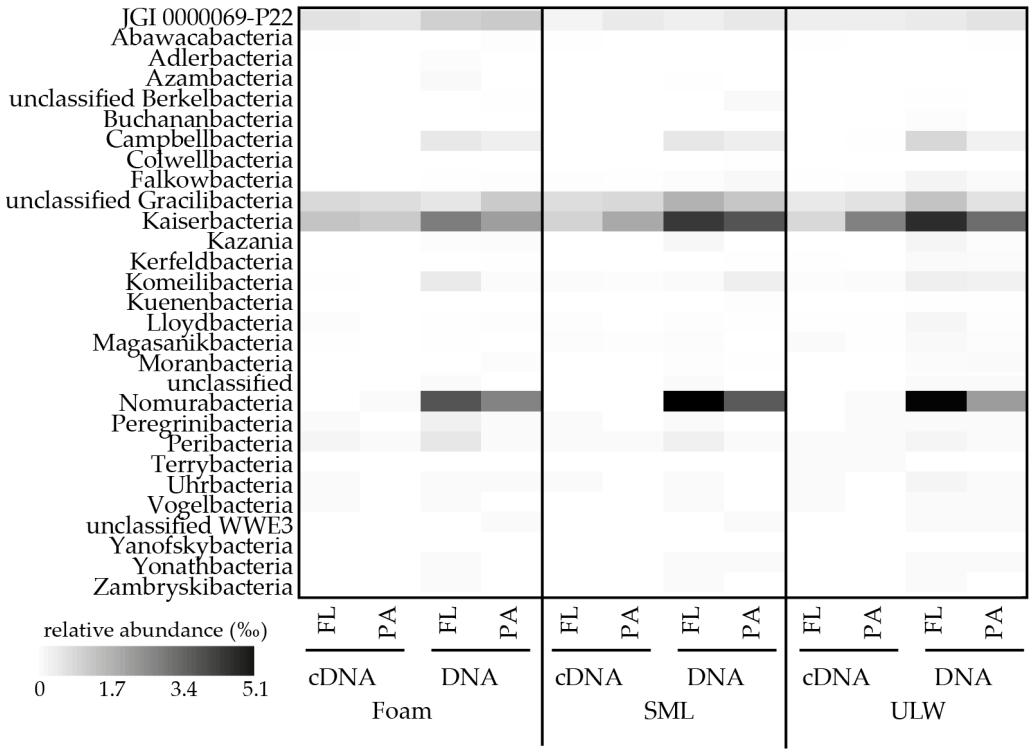


**Figure 3.** Heatmap showing relative abundance (‰) of patescibacterial candidate clades for cDNA and DNA-derived amplicons in foam, sea-surface microlayer (SML) and underlying water (ULW). Samples were further divided into free-living (FL) and particle-associated (PA) fractions. Only lineages with >10 reads are shown.

Analysis of the flow cytometry samples, that are unfiltered, revealed an average LNA portion of 38% (±16.8%), 60% (±24.2%), and 56% (±22.1%) in foams, SML and 1 m deep water, respectively (Table A1 in Appendix A, Figure 4). Due to their small genomes, the LNA fraction probably comprised *Cand.* Patescibacteria [34] suggesting a higher abundance in these fractions as detected by amplicon sequencing. However, in addition to *Cand.* Patescibacteria other LNA bacteria like SAR11 might also be present [54], and more detailed analyses will be necessary to estimate the total abundance of *Cand.* Patescibacteria. In addition to the filtration bias, universal 16S RNA primer and protocols do not cover the complete bacterial diversity and up to 20% of environmental bacterial sequences are missing [2,55]. Mismatch analyses of our primer set in ARB SILVA [56] revealed little coverage of the Microgenomates, one of the major orders of *Cand.* Patescibacteria (Figure A1). In SILVA Ref NR 138 a total of 4543 sequences are assigned to the *Cand.* Patescibacteria, of which the primers Bakt_341F/805R [39] used in this analysis matched 2618 of these sequences (58%, Figure A1). Bakt_341F/805R primers cover *Cand.* Parcubacteria (OD1) sufficiently [57], but only a small set (37%) of the *Cand.* Kaiserbacteria can be amplified (Figure A2). Interestingly, *Cand.* Kaiserbacteria were one of the most abundant groups in our study. *Cand.* Gracilibacteria, the most diverse group in our analysis were fairly well covered (81%) next to *Cand.* Peribacteria (Figure A3). Filtration and primer biases cause that the presented *Cand.* Patescibacteria abundances and especially of *Cand.* Kaiserbacteria are probably underestimated. Metagenomic sequencing approaches are advantageous concerning primer biases. However, current sequencing depth in metagenomics only cover the most abundant bacterial lineages and has only limited quantitative information. In addition, the presence of small eukaryotic organisms, like phytoplankton in surface water, causes a high number of non-target metagenomic sequences and an amplification of bacterial DNA therefore seemed feasible in this study.

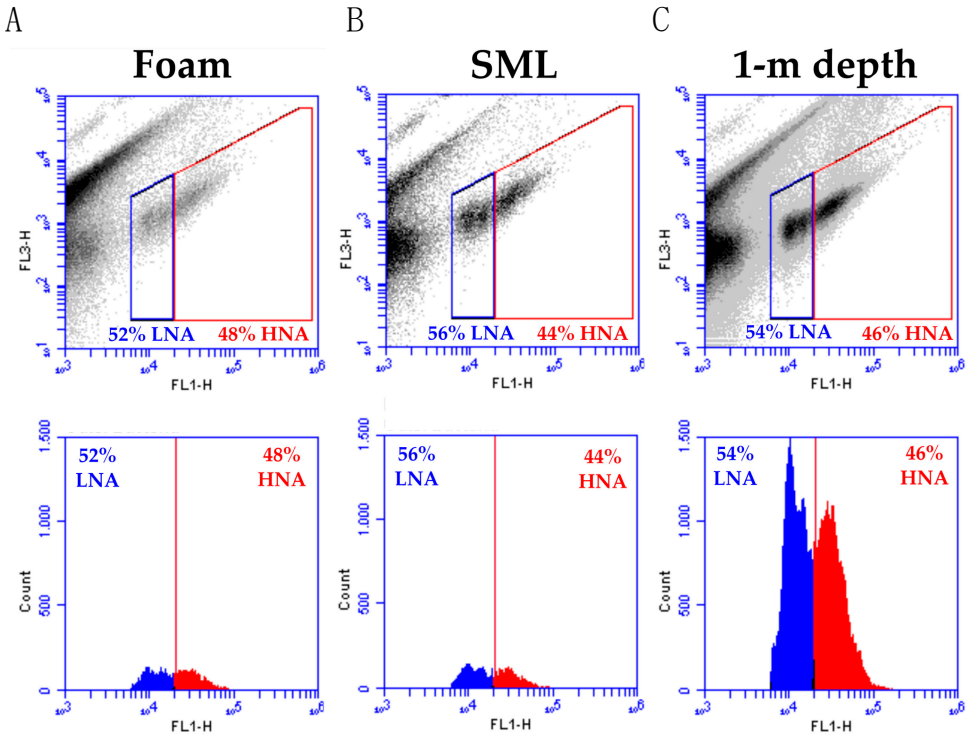


**Figure 4.** Representative example of high nucleic acid (HNA) and low nucleic acid (LNA) cells in (**A**) foam, (**B**) sea-surface microlayer (SML), (**C**) 1-m depth samples from 21st April 2016 (Set 2). Here, more than half of all bacterial cells belong to LNA cells and potentially include *Cand.* Patescibacteria having small genomes.

In marine surface water at 1 m depth (total *Cand.* Patescibacteria abundance 2.1–13.6‰ of assigned bacterial reads), in the SML (total *Cand.* Patescibacteria abundance 2.4–12.6‰ of assigned bacterial reads), and in foams (total *Cand.* Patescibacteria abundance 2.4–9.7‰ of assigned bacterial reads) floating at the air-sea interface *Cand.* Nomurabacteria and *Cand.* Kaiserbacteria as well as JGI 0000069-P22 and unclassified Gracilibacteria were the most abundant phylogenetic lineages (Figure 3). By clustering our sequences with the SILVA Ref seq database and considering the marine or aerosol origin of deposited sequence information, we found similarities of assembled reads from this study to previously detected sequences assigned to *Cand.* Patescibacteria (Table 1, Supplementary Figure S1). Many sequences of marine origin were derived from meromictic waters, sampled in coastal proximity or from a lagoon [58]. Generally, *Cand.* Patescibacteria are frequently described in stratified freshwater lakes [18,57–59], suggesting that they might generally thrive in calmed water, where stagnant conditions could favor facultative symbiotic attachment. This explains the relatively low abundance at the air-sea interface where wind-wave dynamics often lead to profound mixing, which is known to inhibit abundance and activity of certain SML-populating bacteria [37]. However, other studies reported that *Cand.* Patescibacteria are quite responsive and adaptive to changing environments [10] a characteristic favoring their presence at the air-sea boundary. In our study, more OTUs were present in the DNA compared to cDNA-based samples (Figure 2), which is supported by statistical analysis (Table A2). For instance, the number of patescibacterial OTUs was significantly different for DNA-based amplicons of the free-living 1 m deep fractions compared to all cDNA-derived samples from all three habitats (Tukey's HSD, maximum $p$-value = $1.60 \times 10^{-3}$). The minor detection of *Cand.* Patescibacteria among the cDNA-derived amplicons suggests that most *Cand.* Patescibacteria are rather senescent and have an inactive lifestyle. This especially applies to *Cand.* Nomurabacteria, which were more abundant (range 2.0–5.1‰) based on 16S rRNA gene-based amplicons but hardly detectable among rRNA-based amplicons.

**Table 1.** Sequences related to *Cand.* Patescibacteria of marine surface water or atmospheric origin. Sequences were detected based on phylogenetic clustering with samples from this study.

| NCBI Accession # of Sequences from This Study | NCBI Accession # of Phylogenetic Neighbor from Surface Water/Aerosol | Sequence Name | Origin | Phylogenetic Affiliation | Reference |
|---|---|---|---|---|---|
| MW167728, MW167751 | HQ691922 | Uncultured bacterium, 1327, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | JGI 0000069−P22 | [60] |
| MW167732, MW167737, MW167759, MW167763 | HQ691923 | Uncultured bacterium, 1317, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | JGI 0000069−P22 | [60] |
| MW167705, MW167707, MW167722 | HQ691924 | Uncultured bacterium, 1276, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Peregrinibacteria | [60] |
| MW167672, MW167678, MW167694 | HQ691925 | Uncultured bacterium, 1288, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Peribacteria | [60] |
| MW167669, MW167680, MW167690 | HQ691926 | Uncultured bacterium, 1318, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Peribacteria | [60] |
| MW167660, MW167688, MW167703 | HQ691927 | Uncultured bacterium, 1311, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Peribacteria | [60] |
| MW167675, MW167687, MW167717 | HQ691928 | Uncultured bacterium, 1323, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Buchananbacteria | [60] |
| MW167683, MW167684, MW167710, MW167736 | HQ691929 | Uncultured bacterium, 1317, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Moranbacteria | [60] |
| MW167686, MW167713 | HQ691930 | Uncultured bacterium, 1328, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Magasanikbacteria | [60] |
| MW167715, MW167719, MW167721 | HQ691931 | Uncultured bacterium, 1359, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Magasanikbacteria | [60] |
| MW167673, MW167689 | HQ691932 | Uncultured bacterium, 1323, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Komeilibacteria | [60] |
| MW167674, MW167704, MW167711 | HQ691933 | Uncultured bacterium, 1333, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Komeilibacteria | [60] |
| MW167671, MW167699 | HQ691934 | Uncultured bacterium, 1395, stratified lagoon | Clipperton Island atoll, North Pacific Ocean, Meromictic lagoon, marine | *Cand.* Falkowbacteria | [60] |
| MW167663, MW167666 | AACY023814357 | Marine metagenome, 1412, predominantly from surface water marine samples | Surface water samples, off the coast of Bermuda, marine | *Cand.* Falkowbacteria | [61] |
| MW167720 | AACY023758110 | Marine metagenome, 1295, predominantly from surface water marine samples | Surface water samples, off the coast of Bermuda, marine | *Cand.* Kuenenbacteria | [61] |
| MW167725, MW167733, MW167749 | AACY023749576 | Marine metagenome, 1409, predominantly from surface water marine samples | Surface water samples, off the coast of Bermuda, marine | JGI 0000069−P22 | [61] |

**Table 1.** *Cont.*

| NCBI Accession # of Sequences from This Study | NCBI Accession # of Phylogenetic Neighbor from Surface Water/Aerosol | Sequence Name | Origin | Phylogenetic Affiliation | Reference |
|---|---|---|---|---|---|
| MW167667, MW167676, MW167679, MW167691 | AACY020292957 | Marine metagenome, 1318, predominantly from surface water marine samples | Surface water samples, off the coast of Bermuda, marine | *Cand.* Kaiserbacteria | [61] |
| MW167670, MW167692, MW167695, MW167696 | AACY023772748 | Marine metagenome, 1245, predominantly from surface water marine samples | Surface water samples, off the coast of Bermuda, marine | *Cand.* Gracilibacteria | [61] |
| MW167731, MW167744, MW167765 | GU235593 | Uncultured marine bacterium, 1328, Antarctic sea water collected from 5m | Antarctic sea water collected from 5 m, coastal surface waters at Palmer Station, on the west coast of the Antarctic Peninsula | JGI 0000069−P22 | [62] |
| MW167727, MW167734, MW167735, MW167747, MW167748, MW167756 | GU234860 | Uncultured marine bacterium, 1316, Antarctic sea water collected from 5m | Antarctic sea water collected from 5 m, coastal surface waters at Palmer Station, on the west coast of the Antarctic Peninsula | JGI 0000069−P22 | [62] |
| MW167726, MW167740 | LN681284 | Bacterium RFB D08, 1233, marine alga | Ria Formosa tidal pools close to the Ramalhete Marine Station, marine | JGI 0000069−P22 | [63] |
| MW167668, MW167685, MW167700 | FJ744790 | Uncultured bacterium, 1397, surface water at the UGA Marine Institute | Coastal water samples were collected high tide from Sapelo Island, GA, marine | *Cand.* Gracilibacteria | [64] |
| MW167677, MW167701, MW167716 | DQ269061 | Uncultured bacterium, 1342, surface of marine macro−alga | One of three sampling sites, Sydney, Australia, marine | *Cand.* Gracilibacteria | [65] |
| MW167739, MW167743, MW167757 | FJ826108 | Uncultured marine bacterium, 1418, filtered surface sea water | Filtered surface sea water in the decay period after diatom bloom in the Yellow Sea, marine | *Cand.* Peregrinibacteria | [66] |
| MW167739, MW167743, MW167757 | FJ826198 | Uncultured marine bacterium, 1442, filtered surface sea water | Filtered surface sea water in the decay period after diatom bloom in the Yellow Sea, marine | *Cand.* Peregrinibacteria | [66] |
| MW167681 | KU578668 | Uncultured bacterium, 1369, ocean water | Marine | *Cand.* Magasanikbacteria | unpublished |
| MW167681, MW167697, MW167702, MW167712 | FLOH01000114 | Marine metagenome, 1479, water | Marine | *Cand.* Magasanikbacteria | not specified |
| MW167664, MW167714, MW167718 | CEVN01160041 | Marine metagenome, 1440, saline water (ENVO:00002010), including plankton (ENVO:xxxxxxxx) | Marine | *Cand.* Kaiserbacteria | not specified |
| MW167661, MW167665, MW167698 | JQ197106 | Uncultured bacterium, 1330, seawater; next to dolphin E | Marine | *Cand.* Nomurabacteria | [67] |
| MW167693, MW167708, MW167709 | JQ198499 | Uncultured bacterium, 1329, seawater; next to dolphin K | Marine | *Cand.* Nomurabacteria | [67] |
| MW167745, MW167746, MW167753 | JN981903 | Uncultured beta proteobacterium, 1495, aerosols from orbal oxidation ditch in a municipal WWTP | Aerosols from orbal oxidation ditch in a municipal WWTP | *Cand.* Gracilibacteria | [68] |

In contrast to other studies reporting on >20% *Cand.* Patescibacteria in the 0.2 µm fraction for a peatland permafrost thaw lake [16] or in the suboxic hypolimnion of boreal lakes [57], we detected a maximum relative abundance of 1.2–1.4% of *Cand.* Patescibacteria in SML and surface water sample filtered onto a 0.2 µm pore size filter membrane. Since marine surface water, the SML and foams represent aerobic environments, and despite facultative anaerobic bacteria inhabiting the SML [69], the *Cand.* Patescibacteria found in our study are most likely aerobic or at least aerotolerant. Considering these differences in abundance, *Cand.* Patescibacteria are presumably better adapted to freshwater ecosystems and low oxygen environments. The ultrasmall *Cand.* Patescibacteria were also detected on >3 µm filter membranes (Figure 3). This suggests that members of this phylum may also adhere to particles or are symbiotic with organisms on particles. Particles are especially enriched in the SML and foams [28,70,71] and could serve *Cand.* Patescibacteria as a food source. Moreover, aggregates can form low oxygen microenvironments [72,73] allowing *Cand.* Patescibacteria to thrive, though these anoxic microzones might be too short-lived for slow-growing anaerobic microorganisms to take advantage [73]. Our results show that *Cand.* Kaiserbacteria and *Cand.* Gracilibacteria were metabolically active based at the time of their detection as their respective 16S rRNAs were detected and thus represent potential candidates for living in anoxic particles. However, the proposed restricted biosynthetic capabilities may also favor a symbiotic lifestyle [20]. Searching the SILVA Ref 138 database for related sequences revealed that several sequences of *Cand.* Patescibacteria were previously found on microbial communities associated with marine algae, e.g., *Cand.* Gracilibacteria (Table 1). Since algae are integral parts of marine foams in the >3 µm fraction [74], and can be trapped within them [75], members of *Cand.* Patescibacteria may therefore be symbionts of eukaryotic hosts as described earlier [76].

*Cand.* Patescibacteria have been shown to scavenge organic compounds, e.g., lipids [10,77] or nucleotides from external sources and other organisms [10,78], because they lack the required metabolic pathways for de novo biosynthesis. In addition, Vigneron, Cruaud, Langlois, Lovejoy, Culley and Vincent [16] suggested that they have the metabolic capacity for amylose and cellulose degradation since the group of glycoside hydrolase enzymes are part of their metabolism. This supports their role in the degradation of refractory organic matter and, therefore, in the marine carbon cycling. Saccharibacteria, which have cultivated representatives [79,80], were predicted to utilize glucose, amino acids and plant-derived carbon compounds [78,81], substances that are typically found in foams [82–84]. Therefore, despite their low abundance, *Cand.* Patescibacteria might have a crucial role as specialists in degradation processes at the ocean's surface. Degradation of cells and matter could be further enhanced in the SML, because higher bacterial production compared to underlying water promotes lytic viral infections releasing carbon compounds [85]. Strong exposure to solar and ultraviolet radiation is detrimental for bacterioplankton [86]. Even if compensated by some resistance of the bacterioneuston to solar radiation [87], the high irradiance at the air-sea boundary will enhance turnover of cells and release of their content. At the same time, photolysis increases the bioavailability of recalcitrant dissolved organic matter [88]. All these processes support nutrient-scavenging organisms such as *Cand.* Patescibacteria, which heavily rely on external sources for all sorts of molecular building blocks, especially if these ultramicrobacteria do not parasitize a host [6]. In the marine realm, genomic and proteomic approaches combined with cultivation attempts and microscopic methods, e.g., by using organism-specific in situ probing, would be the best approaches to study *Cand.* Patescibacteria, and most knowledge on the clades detected in this study originate from genome-resolved metagenomics. Cultivation attempts of *Cand.* Patescibacteria have been rarely successful for many reasons such as their complicated interactions with multiple eukaryotic hosts [76], lethality to the host [89] or uncharacterized host ranges [90]. In addition, advanced techniques at the single-cell level such as reverse genomics have to be considered for the establishment of pure cultures [80].

Marine foams and the SML are both enriched in organic matter and nutrients [91–93], but can also be considered as challenging habitats due to accumulating pollutants (reviewed by Wurl and Obbard [91,94]) and prevailing effects of meteorological impacts [48,95]. Transport of living and

non-living matter to the air-sea interface is mainly achieved by rising bubbles [96], mixing processes, or the result of atmospheric deposition [97]. At the same time, the SML is a known source for aerosolization of viruses and bacteria to the atmosphere [98] as reviewed by Rahlff [99]. Aerosolization from the SML seems to be very taxon-specific [100]. Therefore, depending on their own and/or their symbiotic partners aerosolization capacities, *Cand.* Patescibacteria might be transferred from 1 m depth to the SML by rising bubbles and from the SML to sea-spray aerosols. Observations on many rare and uncultured OTUs among airborne bacteria in a coastal region of the Baltic Sea [101], and higher contributions of less abundant seawater bacterial taxa being selectively transferred to marine aerosols [102] make signatures of *Cand.* Patescibacteria in marine aerosols very likely. In fact, preliminary data indicate the presence of *Cand.* Perigrinibacteria and *Cand.* Abscondibacteria in aerosol samples, which were artificially generated in a tank experiment during the EMB184 cruise using Baltic Sea surface water (unpublished data). Previous studies showed that aerosols produced by rotating brushes in a wastewater treatment plant were associated with a high portion of uncultured bacteria [68] including a sequence assigned to *Cand.* Gracilibacteria (Table 1). Furthermore, *Cand.* Patescibacteria were recently found in aerosols in the lower atmosphere of the Southern Ocean (7.8%) [31], among aerosolized bacterial loads emitted by a green wall (dominated by the order Saccharimonadales (56.6%)) [103], in blowing Arctic snow (0.2% of OD1) [104], snow layers over sea ice (≤0.3% of TM7) [105], and Arctic air with terrestrial source (24% TM7) [106] and thus seem to potentially resist environmental conditions of the troposphere. Considering the usually low biomass associated with aerosol samples and the often-low portions of *Cand.* Patescibacteria in bacterial communities of marine surface waters, *Cand.* Patescibacteria likely remain underexplored and highly elusive components of the atmosphere. These ultra-small bacteria are rarely the target of sequencing efforts of marine surface water applying filtration protocols on pore sizes >0.2 µm and, if found, they are often not further classified [15,31]. Thus, here we can only speculate that *Cand.* Patescibacteria might experience transfer to the atmosphere from SML via sea spray aerosols, while their presence in foams suggests easy propagation to terrestrial ecosystems as foams get frequently dispersed to beaches by strong winds [107,108].

## 4. Conclusions

Uncultivated bacteria of the *Cand.* Patescibacteria, also referred to as Candidate phyla radiation, were detected in small (<1.3%) but probably underestimated quantities at the air-sea boundary (in the SML and foams) as well as in 1 m deep seawater. These findings support previous sequencing results on a diverse *Cand.* Patescibacteria bacterial community in the upper oceanic water column. Their peculiar lifestyle as symbionts might influence microbial communities and carbon cycling in the surface ocean, and by being further dispersed from these habitats, *Cand.* Patescibacteria might easily end up in the atmosphere and on land. Future work pursuing in situ localization combined with genome analyses as well as cultivation attempts are required to achieve a deeper understanding of the life strategies and underlying metabolic capacities of *Cand.* Patescibacteria at the ocean's surface.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4433/11/11/1214/s1, Figure S1: Neighbor joining tree of *Candidatus* Patescibacteria sequences obtained from this and previous studies.

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## Appendix A

**Table A1.** Percentage (%) of high nucleic acid (HNA) and low nucleic acid (LNA) in different sample sets. ULW = underlying water (refers to 1-m depth), SML = sea-surface microlayer.

| Sample | %LNA | %HNA |
|---|---|---|
| ULW_21st_April_2016_set1 | 55.0 | 45.0 |
| SML_21st_April_2016_set1 | 76.9 | 23.1 |
| Foam_21st_April_2016_set1 | 42.5 | 57.5 |
| ULW_21st_April_2016_set2 | 53.7 | 46.3 |
| SML_21st_April_2016_set2 | 55.8 | 44.2 |
| Foam_21st_April_2016_set2 | 52.0 | 48.0 |
| ULW_19th_May_2016_set1 | 63.1 | 36.9 |
| SML_19th_May_2016_set1 | 62.5 | 37.5 |
| Foam_19th_May_2016_set1 | 32.5 | 67.5 |
| ULW_19th_May_2016_set2 | 65.0 | 35.0 |
| SML_19th_May_2016_set2 | 60.0 | 40.0 |
| Foam_19th_May_2016_set2 | 24.9 | 75.1 |
| ULW_19th_July_2016_set1 | 50.3 | 49.7 |
| SML_19th_July_2016_set1 | 49.7 | 50.3 |
| Foam_19th_July_2016_set1 | 39.8 | 60.2 |
| ULW_19th_July_2016_set2 | 51.6 | 48.4 |
| SML_19th_July_2016_set2 | 52.8 | 47.2 |
| Foam_19th_July_2016_set2 | 39.2 | 60.8 |

**Table A2.** Results of statistical analysis for Figure 2. There was a significant difference between sample medians (Kruskal–Wallis Test, chi-squared: 36.14, $p$-value = $1.6 \times 10^{-4}$). In this table, the upper diagonal reflects $p$-values after Tukey HSD test with significant results ($p \leq 0.05$ indicated in orange) and Tukey's Q value in the lower diagonal. FL = free-living, PA = particle-associated, SML= sea-surface microlayer.

| | | | Foam | | | | SML | | | | 1-m depth | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | cDNA | | DNA | | cDNA | | DNA | | cDNA | | DNA | |
| | | | FL | PA | FL | PA | FL | PA | FL | PA | FL | PA | FL | PA |
| **Foam** | **cDNA** | **FL** | | 1.00 | 0.90 | 0.03 | 1.00 | 1.00 | 0.04 | 0.89 | 1.00 | 1.00 | $5.43 \times 10^{-4}$ | 0.97 |
| | | **PA** | 0.73 | | 0.56 | $3.34 \times 10^{-3}$ | 1.00 | 1.00 | 0.01 | 0.48 | 1.00 | 1.00 | $4.19 \times 10^{-5}$ | 0.70 |
| | **DNA** | **FL** | 2.26 | 3.11 | | 0.78 | 0.81 | 0.76 | 0.74 | 1.00 | 0.93 | 0.91 | 0.06 | 1.00 |
| | | **PA** | 5.10 | 6.24 | 2.63 | | 0.02 | 0.01 | 1.00 | 0.56 | 0.07 | 0.02 | 0.77 | 0.34 |
| **SML** | **cDNA** | **FL** | 0.29 | 0.43 | 2.55 | 5.42 | | 1.00 | 0.02 | 0.78 | 1.00 | 1.00 | $2.83 \times 10^{-4}$ | 0.92 |
| | | **PA** | 0.28 | 0.47 | 2.66 | 5.75 | 0.02 | | 0.01 | 0.71 | 1.00 | 1.00 | $1.19 \times 10^{-4}$ | 0.89 |
| | **DNA** | **FL** | 4.97 | 5.97 | 2.71 | 0.35 | 5.26 | 5.52 | | 0.55 | 0.08 | 0.03 | 0.94 | 0.35 |
| | | **PA** | 2.31 | 3.27 | 0.16 | 3.12 | 2.63 | 2.78 | 3.13 | | 0.93 | 0.89 | 0.02 | 1.00 |
| **1-m depth** | **cDNA** | **FL** | 0.04 | 0.63 | 2.13 | 4.70 | 0.23 | 0.22 | 4.64 | 2.16 | | 1.00 | $1.60 \times 10^{-3}$ | 0.98 |
| | | **PA** | 0.14 | 0.93 | 2.24 | 5.28 | 0.45 | 0.45 | 5.10 | 2.31 | 0.18 | | $3.16 \times 10^{-4}$ | 0.98 |
| | **DNA** | **FL** | 7.07 | 8.18 | 4.81 | 2.64 | 7.36 | 7.73 | 2.10 | 5.43 | 6.58 | 7.31 | | 0.01 |
| | | **PA** | 1.87 | 2.80 | 0.61 | 3.61 | 2.18 | 2.30 | 3.58 | 0.50 | 1.75 | 1.83 | 5.87 | |

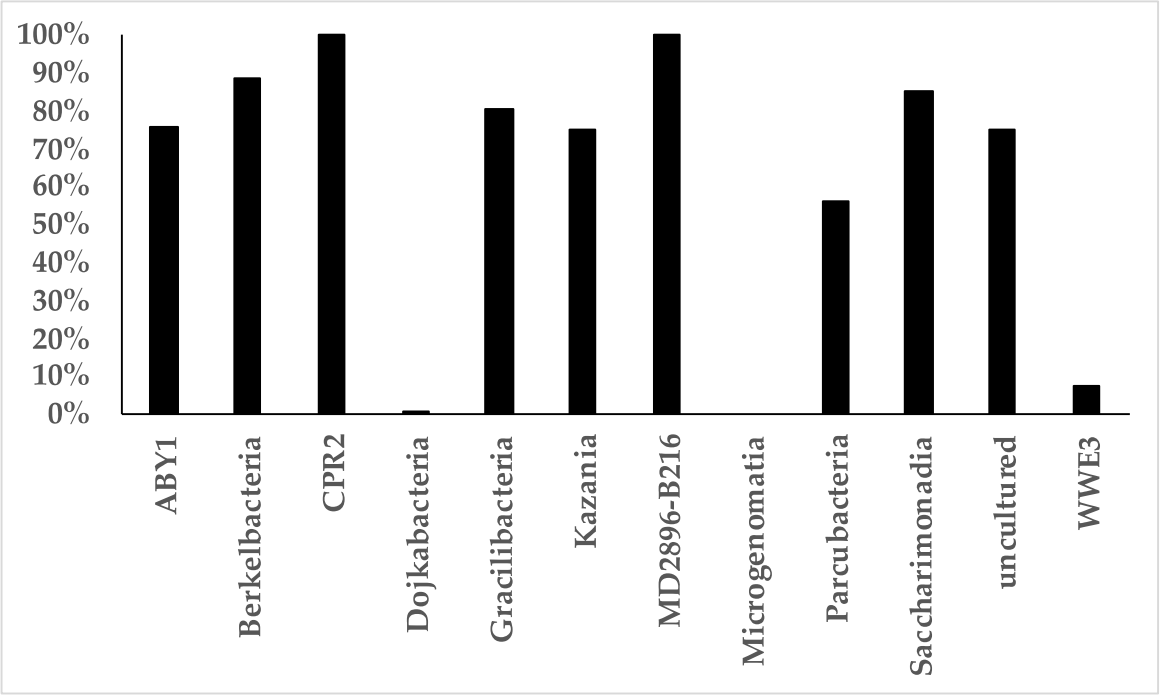


**Figure A1.** Coverage of primer set Bakt_341F/805R of various *Cand.* Patescibacteria clades and orders.

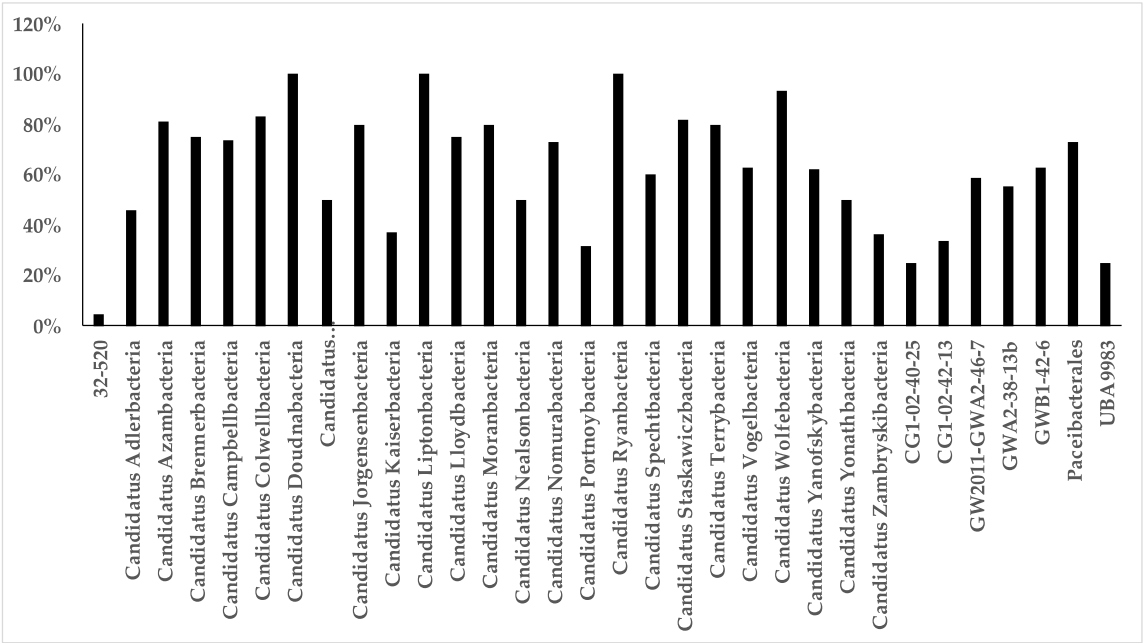


**Figure A2.** Coverage of primer set Bakt_341F/805R for various *Cand.* Parcubacteria clades (based on SILVA 138).

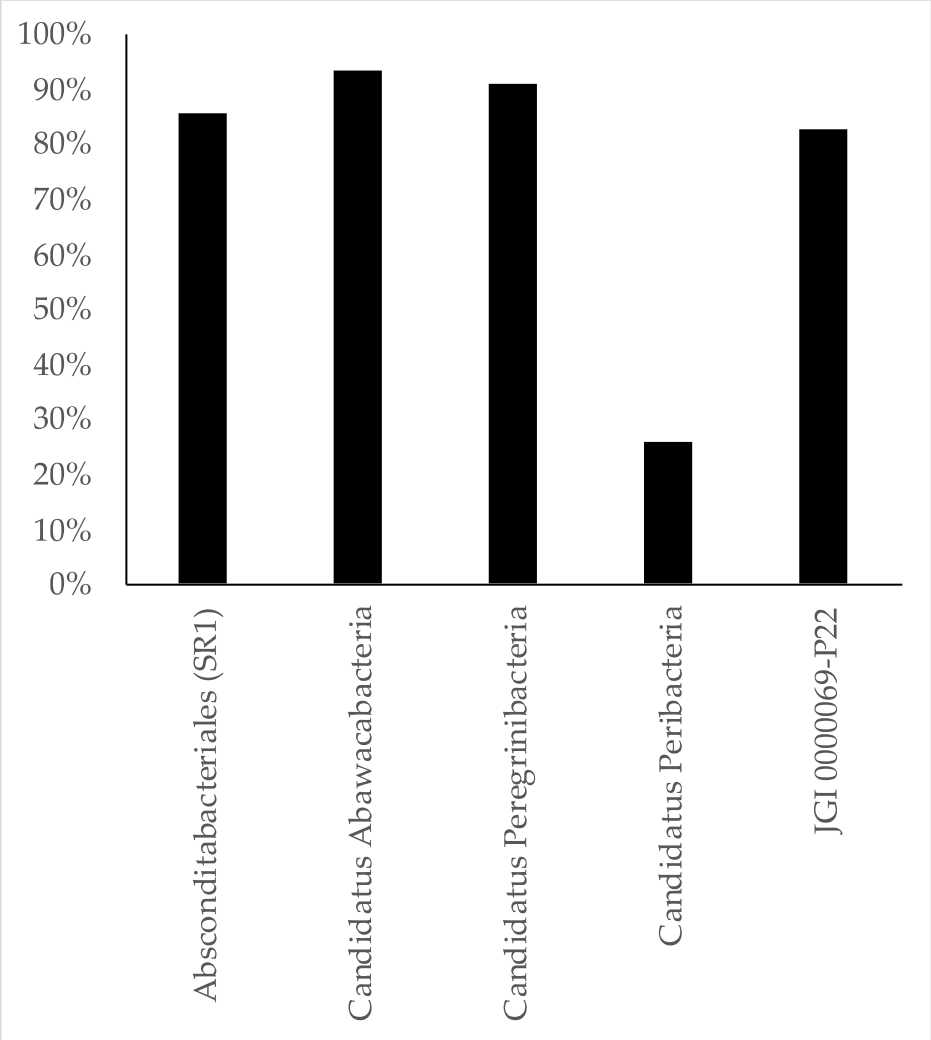


**Figure A3.** Coverage of primer set Bakt_341F/805R for *Cand.* Gracilibacteria clades.

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