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Diversity and abundance of "*Pelagibacterales*" (SAR11) in the Baltic Sea salinity gradient

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ABSTRACT

The candidate order "*Pelagibacterales*" (SAR11) is one of the most abundant bacterial orders in ocean surface waters and, periodically, in freshwater lakes. The presence of several stable phylogenetic lineages comprising "*Pelagibacterales*" correlates with the physico-chemical parameters in aquatic environments. A previous amplicon sequencing study covering the bacterial community in the salinity gradient of the Baltic Sea suggested that pelagibacteral subclade SAR11-I was replaced by SAR11-IIIa in the mesohaline region of the Baltic Sea. In this current study, we investigated the cellular abundances of "*Pelagibacterales*" subclades along the Baltic Sea salinity gradient using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). The results obtained with a newly designed probe, which exclusively detected SAR11-IIIa, were compared to CARD-FISH abundances of the marine SAR11-I/II subclade and the freshwater lineage SAR11-IIIb (LD12). The results showed that SAR11-IIIa was abundant in oligohaline–mesohaline conditions (salinities 2.7–13.3), with maximal abundances at a salinity of 7 (up to 35% of total *Bacteria*, quantified with a universal bacterial probe EUB). As expected, SAR11-I/II was abundant (27% of EUB) in the marine parts of the Baltic Sea, whereas counts of the freshwater lineage SAR11-IIIb were below the detection limit at all stations. The shift from SAR11-IIIa to SAR11-I/II was confirmed in the vertical salinity gradient in the deeper basins of the Baltic Sea. These findings were consistent with an overlapping but defined distribution of SAR11-I/II and SAR11-IIIa in the salinity gradient of the Baltic Sea and suggested the adaptation of SAR11-IIIa for growth and survival in mesohaline conditions.

Introduction

Ecological research involves the investigation of species distributions as a function of measurable environmental parameters. The introduction of high-throughput sequencing methods has also allowed detailed studies of microbial communities present within large environmental gradients, and thus the determination of several environmental parameters that influence bacterial community composition. Among these, salinity is one of the most important factors in structuring microbial communities and their distribution [12,18]. Indeed, marine and freshwater bacterial communities differ considerably, suggesting that within a single bacterial order transitions between these environments are evolutionarily rare [17]. One such exception is the candidate order "*Pelagibacterales*" (SAR11), as its freshwater and marine subclades have only recently diverged [36] and they are found abundantly in their respective environments [3,20]. The "*Pelagibacterales*" belong to the subphylum *Alphaproteobacteria*, with the proposed candidate genus "*Candidatus* Pelagibacter" being placed in the subclade SAR11-I [11]. The order currently consists of five different subclades [11] with up to 18% sequence divergence of the 16S rRNA gene. The most divergent "subclade V" is polyphyletic and can therefore be excluded from this order [34].

Based on an analysis of concatenated phylogenetic marker genes, subclade SAR11-III is monophyletic within the "*Pelagibacterales*" [11,36]. It is divided into SAR11-IIIa, consisting predominately of sequences derived from mesohaline environments, and the freshwater lineage SAR11-IIIb (LD12) [3,11]. SAR11-IIIa encompasses two, not yet described, phylogenetically distinct strains, each of which displays a 12% 16S rRNA sequence difference with "*Candidatus* Pelagibacter ubique" (subclade SAR11-I). Of the two strains, strain HIMB114 has been isolated from Kaneohe Bay [11], and strain IMCC9063 from brackish waters in Svalbard [23]. The sister lineage SAR11-IIIb (LD12) has mostly

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been found in freshwater systems [3,31] and, although it currently contains no cultivated representatives, it has several single-cell genomes [36].

The stable phylogenetic lineages of the "*Pelagibacterales*" exhibit distinct spatio-temporal abundance patterns in the water column [5,16,20–22]. An amplicon sequencing study of samples from the Baltic Sea demonstrated shifts in the relative abundance of amplicon sequencing reads from subclade SAR11-I to subclade SAR-IIIa in the transition from marine to oligohaline conditions [12]. Together with the predominance of SAR11-IIIa sequences in mesohaline environments, these observations suggested that SAR11-IIIa is primarily adapted for growth and survival in mesohaline conditions, in contrast to marine SAR11-I/II and freshwater SAR11-IIIb. However, the biases of amplicon sequencing, a PCR-based method, hinder reliable quantifications of taxa abundances [2,29,33]. Thus, the goal of this study was to use catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) to examine quantitatively the shifts between SAR11-I/II, SAR11-IIIa, and SAR11-IIIb in the natural salinity gradient of the Baltic Sea. The results supported changes in the "*Pelagibacterales*" community structure based on salinity and showed that SAR11-IIIa was highly abundant in mesohaline conditions.

Materials and methods

Samples were taken in June and July 2008 in the Baltic Sea during cruise MSM0803 with the research vessel *Maria S. Merian*. Water samples were collected using a conductivity/temperature/depth (CTD) SeaBird 911 sensor connected to a rosette of 24 10-L bottles. Inorganic nutrients and oxygen were analyzed on board with standard methods [10]. Simultaneously, water samples for CARD-FISH were fixed with formaldehyde (2%, v/v) at room temperature for at least 1 h, filtered on white polycarbonate filters (0.2 μm, GTTP, Millipore), and stored at −80 °C until use.

All 16S rRNA gene sequences assigned to "*Pelagibacterales*" in the SILVA database SSU_NR_117 [30] with a sequence length >1000 bp and a pintail score >75 were used to calculate a base tree of "*Pelagibacterales*" based on 1483 unambiguously aligned sequence positions. A 16S rRNA gene sequence assembled from a Baltic Sea metagenome [8] and sequences from *Rickettsia* ("root") were also added. Representatives of the resulting 7118 sequences were selected using a 1% cut-off in the "cluster detection" tool of ARB 6 [19]. This yielded 913 sequences that were used in a maximum likelihood analysis (PHYML, GTR rate distribution model) provided in the ARB package and employing the same filter as described above. The phylogenetic tree was visualized in FigTree (v1.4.0; http://tree.bio.ed.ac.uk/software/figtree/), with the subclades collapsed and assigned based on the nomenclature of Grote et al. [11] (Fig. 1). The complete phylogenetic tree has been deposited at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S16516). The topology of the SAR11-III subclade was tested separately by neighbor-joining with bootstrapping (1000 bootstraps) and parsimony analysis (DNAPARS), and the nodes not supported by all analyses were shown as multifurcations [25]. The maximum likelihood tree provided the basis for assigning the published "*Pelagibacterales*" probes SAR11-441R [20] and LD12-121 [31] (Fig. 1) and for designing a new probe, SAR11-440b (5′-TACAGTCATTATCTTTCCCGACA-3′), using the ARB ProbeMatch tool. The newly designed probe was evaluated using the TESTprobe function of SILVA [30] on SSU NR_117_PARC (Table S1).

In this study, 35 samples from 19 stations were analyzed by CARD-FISH (Table S2, Fig. S1) that was carried out using a modification of the protocol of Pernthaler et al. [26]. In brief, samples on membrane filters were first treated with lysozyme (37 °C for 60 min) and then with achromopeptidase (37 °C for 30 min) [32]. Next, they were hybridized for 2–3 h at 35 °C (50 μmol probe μL^{-1}) and stained with DAPI (4′,6-diamidino-2-phenylindole). After hybridization, the samples were washed in a washing buffer with the same formamide concentration as before at 37 °C for 30 min. Since the signal intensities were low, and in order to improve the stringency of probe 440b, an unlabeled helper probe, 440b-help (5′-AAAGAGCTTTACAACCCAAAGG-3′), was added [9]. The stained filter sections were evaluated using an Axio Imager.M1 microscope (Carl Zeiss) with a 100× Plan Apochromat objective equipped with a Colibri LED and Zeiss filter set 15 (BP546/12; FT580; LP590) for the detection of DAPI-stained cells (wavelength of 365 nm) and hybridized cells (wavelength of 450–490 nm). At least 400 cells (10–15 grids) per sample were counted for each filter. Bacterial abundances in the field samples were estimated using EUBmix (EUBI, EUBII and EUBIII) [1,7] and signals identified by NonEUB [35] were subtracted (always <1% of total bacterial counts). The Spearman rank correlation for environmental parameters was calculated using the R (www.r-project.com) vegan package [24] in order to relate the abundances of the "*Pelagibacterales*" subclades to biotic and abiotic parameters (Table S3). The maps of the vertical (Fig. 2) and horizontal (Fig. S1) salinity gradients were generated using Ocean Data View (Schlitzer, R., Ocean Data View, 2014, http://odv.awi.de) based on the salinity data from Herlemann et al. [12].

Results and discussion

Phylogenetic analysis based on different algorithms of the 913 high-quality "*Pelagibacterales*" sequences confirmed that SAR11-III was a monophyletic subclade. The lineage SAR11-IIIb (LD12) was consistently separated from SAR11-IIIa (Fig. 1). Within SAR11-IIIa, most sequences recovered from brackish environments, such as

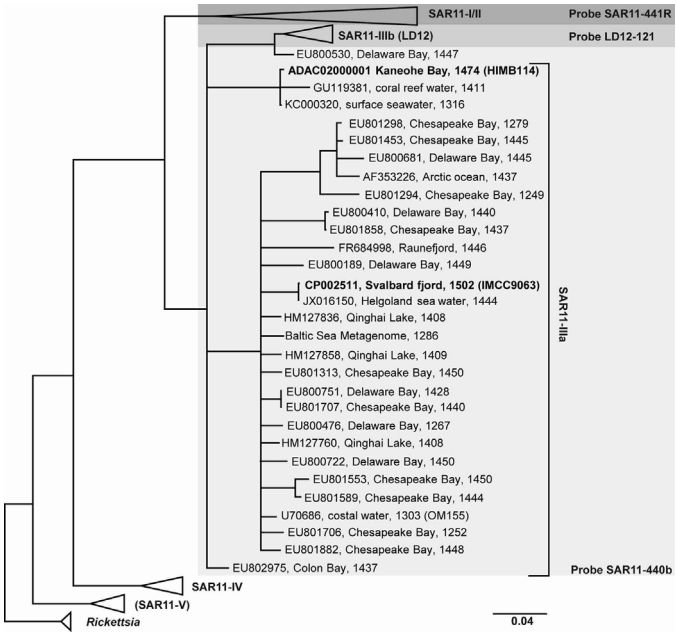

Fig. 1. Maximum likelihood (PHYML) tree of 913 representative sequences of "*Pelagibacterales*" taken from SILVA_NR_117. The stability of the tree was tested by neighbor-joining and parsimony (DNAPARS) analyses, and the nodes not supported by all analyses are shown as multifurcations. The assignment of the subclades is based on reference sequences from Grote et al. [11]. Only subclade SAR11-IIIa is shown in detail, and the cultivated strains IMCC9063 and HIMB114 are indicated in bold. The gray background color indicates the coverages of the probes used in this study: LD12-121 [31], SAR11-441R [20], and SAR440b (this study). The original sequence definitions in SILVA were replaced with a consistent nomenclature including accession number, isolation source, and sequence length (in base pairs). The scale bar represents 4% nucleotide divergence.

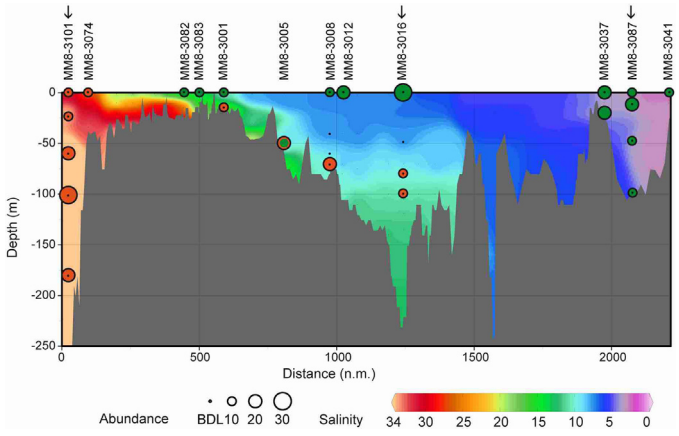

Fig. 2. Distribution of SAR11-I/II (orange dots) and SAR11-IIIa (green dots) for representative stations along the horizontal and vertical salinity gradients of the Baltic Sea. Detailed for the sampling stations are found in Table S2. Arrows mark the vertical profiles of the marine (MM8-3101), mesohaline (MM8-3016), and oligohaline (MM8-3087) stations in Fig. S2. The dot diameters reflect the relative cellular abundances of detected cells counted after catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). BDL: below the detection limit. The map was generated using Ocean data view (Schlitzer, R., Ocean Data View, 2014, http://odv.awi.de).

Chesapeake Bay [14], the Delaware Estuary [6], Saanich Inlet (acc. no. GQ347944), the Baltic Sea [8], and saline Qinghai Lake (acc. no. HM127836), were affiliated to strain IMCC9063, whereas SAR11-IIIa sequences recovered from coastal environments were related to strain HIMB114 (Fig. 1). This suggested the presence of both a coastal and a mesohaline phylogenetic lineage within SAR11-IIIa.

For the newly designed probe SAR11-440b, TESTprobe identified 521 sequences without mismatches (Table S1) and 547 sequences allowing one mismatch. Of the 521 sequences, 56 had a low pintail score (<75), indicating potential sequence anomalies or chimeras [2], and they were therefore excluded. Among the remaining 465 (486 allowing one mismatch) sequences, 319 (324 allowing one mismatch) had a sequence length >1000 bp. These sequences represented all sequences assigned to SAR11-IIIa in Fig. 1. TESTprobe identified one outlier affiliated to SAR11-II (EU800153) and one sequence that fell close to SAR11-IIIb (Fig. 1, acc. no. EU800530). The SAR11-IIIb sequence was, however, derived from the Delaware Estuary and it was not covered by probe LD12-121 (Fig. 1). Both the probe and the helper probe were tested at different formamide concentrations (40–55%), and optimal hybridization conditions were obtained with 50% formamide. Under these conditions, the signals of cells from the negative control "*Candidatus* Pelagibacter ubique" strain HTCC1062 (three mismatches with SAR11-440b; Table S4) and from *Bosea vestrisii* DSM 21229 (two mismatches with SAR11-440b; Table S4) accounted for <1% of the DAPI counts.

The new probe, SAR11-440b, revealed the high abundance (10.8%–34.6% of EUB-positive cells, Table S2) of SAR11-IIIa in surface waters within a salinity range of 2.7–13.3 (Fig. 2, green dots). The highest abundance (34.6% of EUB-positive cells) occurred in surface samples obtained at a salinity of approximately 7.0. At the transitions between oligohaline and mesohaline conditions (salinities 2.6–3.7), abundances were still high (12.6–20.2% of EUB counts; Fig. 2). At a salinity of 28.9, the abundance of SAR11-IIIa in Baltic Sea surface water was below the detection limit of the method (<1% of EUB counts). Spearman rank analysis showed a significant negative correlation between SAR11-IIIa abundance and salinity ($\rho = -0.8$; $n = 31$; $p < 0.01$) (Table S3). Quantification using probe SAR11-441R, which covers the SAR11-I/II group (Fig. 1), revealed a positive Spearman rank correlation between SAR11-I/II abundance and salinity ($\rho = 0.7$; $n = 31$; $p < 0.01$) (Table S3). The highest abundance of SAR11-I/II (26.6% of EUB-positive cells) was detected at a salinity of 35.2 (Fig. 2, orange dots) but it decreased significantly when the salinity fell below 9 (<5% of EUB-positive cells). The abundance of the freshwater subclade SAR11-IIIb (LD12) was investigated using probe LD12-121 on samples obtained at a low salinity (2.6–3.6). The abundance of LD12-121-positive cells was below the detection limit (<1% of total bacterial counts) in all tested samples (Table S2).

The vertical distributions of SAR11-I/II and SAR11-IIIa were compared at three stations representing different salinity regimes (Fig. 2, Fig. S2). At the marine station (Kattegat, Fig. 2 MM8-3101, Fig. S2A, salinity 30–35), the abundance of SAR11-I/II increased along the vertical salinity gradient, with counts ranging from 7.3% of EUB-positive cells at the surface to 26.6% in deeper water. SAR11-IIIa counts at this station were below the detection limit (<1% of EUB counts). At the mesohaline-marine station in the central Baltic Sea (Gotland Deep, Fig. 2 MM8-3016, salinity 7.4–11.3; Fig. S2B), SAR11-IIIa abundance was highest in the surface water (15.3% of EUB counts) but decreased to 2.6% at a depth of 60 m. SAR11-I/II showed the opposite trend at this station, with relative counts increasing from 4.4% at 60 m to 20.2% at 70 m. This increase was concomitant with an increase in salinity, from 8.3 to 11.3. SAR11-I/II became rare (<3.5% of total bacterial counts) in the transition between mesohaline and oligohaline conditions (Bothnian Bay, Fig. 2 MM8-3087, salinity 2.8–3.7; Fig. S2C), whereas SAR11-IIIa still accounted for 12.9% of the total EUB counts at 1 m and even 20.2% at 10 m (salinity 2.8).

A comparison of the amplicon sequencing results from Herlemann et al. [12] and the CARD-FISH data (Fig. S3) showed a significant correlation between the abundance determined by the two methods for both SAR11-IIIa ($R^2 = 0.51$; $p < 0.001$) and SAR11-I/II ($R^2 = 0.64$; $p < 0.001$). However, the relative read abundances detected by amplicon sequencing were on average about one-third lower than the relative cell abundances determined by CARD-FISH. This was in contrast to the results in a study of *Spartobacteria* within the salinity gradient of the Baltic Sea, in which the relative number of amplicon sequencing reads was four times higher than the CARD-FISH counts [4]. Since representative genomes from *Spartobacteria* and "*Pelagibacterales*" contain only one 16S rRNA gene [13,23], factors other than the 16S rRNA copy number must be responsible for the differences between the amplicon sequencing results and those of CARD-FISH. In the case of "*Pelagibacterales*", under-estimation by amplicon sequencing could have been due to a mismatch of the reverse primer [15], which may have reduced the amplification efficiency.

The cellular abundance of "*Pelagibacterales*" determined by CARD-FISH provided support for niche partitioning along the salinity gradient of the Baltic Sea. SAR11-IIIa seemed to be primarily adapted to mesohaline conditions, as shown not only for the surface salinity gradient but for the vertical salinity gradient as well (Fig. 2). Recently, SAR11-IIIb (LD12) was also detected in mesohaline conditions in a southeastern bay (Gulf of Gdansk) of the Baltic Sea [28]. The abundance of SAR11-IIIb was less than that in the respective freshwater environments and was driven by local conditions as well as by hydrological dynamics. However, the presence of metabolically active SAR11-IIIb bacteria in oligohaline–mesohaline waters demonstrates the ability of "*Pelagibacterales*" to readily adapt to new environmental conditions [28]. A metagenome study of "*Pelagibacterales*" in the Baltic Sea showed that sequences from the mesohaline parts of the Baltic Sea clustered with strain IMCC9063, rather than with HIMB114 [8]. These metagenomes suggested that SAR11-IIIa is adapted to the *de novo* synthesis of amino acids, nucleotides and cofactor utilization, in contrast to marine "*Pelagibacterales*" [8]. However, similar to many marine "*Pelagibacterales*", strain IMCC9063 has a relatively small genome (1.28 M bases) and contains genes for a light-dependent proton pump (NCBI Locus Tag: SAR11G3_00893). Therefore, other mechanisms may also account

for the success of SAR11-IIIa in aquatic environments, including osmolyte regulation [8], genomic recombination rates [36], viral lysis [37], and possibly also anti-predator defenses [27].

The results of our cellular quantification of "*Pelagibacterales*" in the salinity gradient of the Baltic Sea reinforced the strong capability of this ubiquitous candidate order to proliferate successfully in aquatic environments, including those characterized by mesohaline conditions, and highlighted the influence of salinity on population structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2014.09.002.

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