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Determination of the Diversity of *Rhodopirellula* Isolates from European Seas by Multilocus Sequence Analysis▽†

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In the biogeography of microorganisms, the habitat size of an attached-living bacterium has never been investigated. We approached this theme with a multilocus sequence analysis (MLSA) study of new strains of *Rhodopirellula* sp., an attached-living planctomycete. The development of an MLSA for *Rhodopirellula baltica* enabled the characterization of the genetic diversity at the species level, beyond the resolution of the 16S rRNA gene. The alleles of the nine housekeeping genes *acsA*, *guaA*, *trpE*, *purH*, *glpF*, *fumC*, *icd*, *glyA*, and *mdh* indicated the presence of 13 genetically defined operational taxonomic units (OTUs) in our culture collection. The MLSA-based OTUs coincided with the taxonomic units defined by DNA-DNA hybridization experiments. BOX-PCR supported the MLSA-based differentiation of two closely related OTUs. This study established a taxon-area relationship of cultivable *Rhodopirellula* species. In European seas, three closely related species covered the Baltic Sea and the eastern North Sea, the North Atlantic region, and the southern North Sea to the Mediterranean. The last had regional genotypes, as revealed by BOX-PCR. This suggests a limited habitat size of attached-living *Rhodopirellula* species.

The biogeography of microorganisms describes the habitat size of the species and the distribution of microorganisms on Earth. The experimental approaches depend on the focus of the studies. Habitats are often analyzed by environmental microbiologists with genetic-fingerprinting techniques, with up to 200 bands or fragments representing the whole community. Although the taxonomic resolution of these operational taxonomic units (OTUs) is limited, the studies revealed a community biogeography (22). Medical microbiologists analyze the alleles of housekeeping genes of microorganisms to gain insight into the epidemiology of pathogens, the population biogeography (2). This strain-specific, fine-scale taxonomic resolution within a species is well suited to observance of recent dispersal events. At the species level, multilocus sequence typing (MLST) and analysis (MLSA), which were developed for intraspecies and intragenus specific studies, respectively, consist of the sequences of several (at least seven) housekeeping gene fragments concatenated to an approximately 5-kilobase alignment (17). Recent MLSA studies revealed its applicability to marine isolates and the analysis of biogeographic patterns: *Alteromonas macleodii* isolates could be grouped in an epipelagic and an abyssal clade (6), and strains of *Pseudomonas aeruginosa* were genetically well separated into groups of coastal and oceanic origin (8). However, for *Salinibacter ruber* strains, biogeographical distinctness was not resolved in an MLSA study but showed allopatry in a metabolic analysis (31). Several studies used MLSA together with DNA-DNA hybridization (DDH) for the delineation of new species, e.g., for *Vibrio* and *Ensifer* spp. (20, 36).

In the biogeography of microorganisms, the experimental proof of a local genetic evolution was first revealed at sample sites that were physically separated by over 18,000 km (39). Large populations and the small size of microbes have been considered as facilitators for dispersal over long distances, eventually establishing cosmopolitan microbial populations. On the other hand, the smallest spatial scale of a microbial species in an open system has not been investigated. Attached-living bacteria disperse only during a distinct, short time span in their lives. This limitation of the dispersal time stimulated this study of the biogeography of *Rhodopirellula baltica* in European seas.

R. baltica is a planctomycete with typical morphological features. The peptidoglycanless bacteria have an intracellular compartmentation: the riboplasm with the nucleoid is separated by a membrane from the surrounding paryphoplasm. Cells attach with a holdfast substance to surfaces or, in culture, to themselves, forming typical rosettes. Proliferation occurs by budding, and offspring cells live free in the water column: they are motile with a flagellum until they settle on the sediment (4).

Seventy recently isolated strains affiliated according to the 16S rRNA gene analysis with *R. baltica* SH1^T as the closest validly described species (40). The 16S rRNA gene sequences do not offer sufficient information at the species level. A dissimilarity of the 16S rRNA genes of more than 3%, recently reduced to 1.3% (34, 35), indicates that the strains under consideration belong to two species. These thresholds yielded in our strain collection, according to an ARB-based calcula-

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TABLE 1. Primers used for MLSA

Gene	Forward primers (f1 and f2)	Reverse primers (r1 and r2)	MLSA amplicon (gene positon)
acsA (RB13264)	ACAACTTGTGGGCTAAACGGAC GCTATGTTGTGTACGGACCTTTT[a]	GTGCCCGCGGTCATTGGAAGT GGAAGCGCCGCCGTGAAGC	1025–1748
fumC (RB7623)	TCGAACCGAACGCGACTCGAT CGCTTACTACGGTGCCCAGACTCA	CACATCACCGGATTGACCTTTC TGACACCACCGGCCAACAACT	496–981
glpF (RB8872)	GTGGAACCATGCATCCGTACG TGGCGGTCAAACGAATACTG	CGGGTAGCAACCCGATCGAG[a] CCTTGTTTCCCTTTGATTGGTAGC	127–606
glyA (RB6215)	CCGAAACGACTCGTCAGCAAG GTGACACCGTGCTGGGATTGG[a]	ACCCAGATCCACAGCGGTGA CAGCATCAGGTGGTTGTCGGTTCC	273–860
guaA (RB8374)	GACATCTCGGCCGAACGCATTG ACAGCGTTTACGAAGAAGGTG	TTTGAACAAGTCTCGCAGAGGTT TTCAAACCCCAATTCTTCAGG	417–1138
icd (RB1593)	TTGGCAGAACTTGGCGAGTTG CCGGTCGGATTTTGGCTGGTTTT	TGGCATCCGCAATTTGCTCCG ATAGCAACGATCGGGAATGA	260–680
mdh (RB7652)	ACATTCCCCGAACCGAAGACAT TTGTCGGAGCCGGAAATGTTGG	CCTTGCGAGTCCGGTCAACG[a] CAGTATGCGGCGACAGGGATGAC	166–631
purH (RB10113)	CCAGCCTTGTGCGAGCGGC ATGTCGCGATCGCTACCAGT	GGAAGAACGCATCGGACGCG GCTCTGCGGCTTTCTTGAT	465–880
trpE (RB7967)	GAAGACCAACGCCAGCCGCT GCAAGCCGCGGCCGAAGAAGT	CGCGGGCCTTGTTGAGGGTT AGGCGTTGCGGGATTCTC	852–1571

[a] Primer is located inside the partial sequence used in the MLSA.

tion, five or eight operational taxonomic units besides the species *R. baltica* (40). For strains with highly identical sequences, whole-genome DDH experiments have to be performed to identify the affiliation to established species. Recently, multilocus sequence analyses have emerged as a possible alternative method. Our strain collection comprised many strains with a 16S rRNA gene sequence very closely related to that of *R. baltica* SH1^T. To gain insight into the genetic identity of the isolates on the species level and the habitat sizes of the species, we developed a multilocus sequence analysis and applied it to the strain collection. The MLSA results were calibrated with a DDH study. The closely related strains were additionally characterized by BOX-PCR, a fingerprinting method (15). Transmission electron microscopy (EM) was performed on some isolates to support the identification as *Planctomycetes* and to visualize morphological differences between strains.

MATERIALS AND METHODS

***Rhodopirellula* strain collection.** The strains included in this study were the type strain of *R. baltica*, SH1^T (DSM 10527^T = IFAM 1310^T = NCIMB 13988^T); a collection of *R. baltica* strains from the Bay of Kiel (obtained from H. Schlesner); and isolates from Europe, Tanzania, and the Philippines (40). Biomass was obtained from liquid cultures or colonies on plates grown on *N*-acetylglucosamine (40).

DNA isolation. Genomic DNA was isolated according to the method of Marmur (18) or with a genomic-DNA isolation spin kit (Qiagen, Hilden, Germany).

MLSA design and performance. Following the suggestions of Maiden and colleagues (17), we developed an MLSA that finally consisted of an alignment of 5,026 bp comprising a concatenate of the partial sequences of the following genes: acetyl coenzyme A synthetase (*acsA*; 724 bp), GMP synthetase (*guaA*; 722 bp), anthranilate synthase component 1 (*trpE*; 720 bp), phosphoribosylaminoimidazole carboxamidase (*purH*; 419 bp), glycerol facilitator uptake protein (*glpF*; 480 bp), fumarate hydratase (*fumC*; 486 bp), isocitrate dehydrogenase (*icd*; 421 bp), serine hydroxymethyltransferase (*glyA*; 588 bp), and malate dehydrogenase (*mdh*; 466 bp) (Table 1). Candidate housekeeping genes were taken from established MLSA schemes for *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bordetella pertussis*, and *Haemophilus influenzae* (http://www.mlst.net; 1). The gene presence as a single copy in the genome of *R. baltica* SH1^T and its position outside of the inversion region between 0.0 and 0.4 Mb (5) were confirmed. Primers were designed in Lasergene 6.0 (DNAStar, Madison, WI) (Table 1), and the primer specificity was verified by BLAST searches against the nucleotide database and the genomes of *R. baltica* and *Blastopirellula marina*. The primers were tested with genomic DNA of the type strain, SH1^T, and strains SH190, SH198, and SH386. Amplifications were performed in a final volume of 50 μl containing 1 to 2 μl of template (approximately 50 to 200 ng DNA), 5 μl of 10× buffer (100 mM Tris [pH 9.0], 500 mM KCl, 1.0% Triton X-100, 20 mM $MgCl_2$, 2 mM dithiothreitol [DTT]), 1 unit of *Taq* polymerase, 1 μl of deoxynucleotide triphosphates (dNTPs) (10 mM), 10 pmol of each primer, and up to 40 μl of H_2O. The cycler program was as follows: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C (ramp, 1°C/s), 3 min at 72°C, and a final elongation for 10 min at 72°C. The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide for quantification. Gradient PCRs determined the optimal annealing temperatures. The amplicons were purified by size exclusion chromatography and sequenced. The reaction mixtures were separated on Applied Biosystems 3130XL Genetic Analyzers. Sequences were manually examined with Applied Biosystems Sequencing Analysis 5.2 and assembled in Lasergene with the assistance of the corresponding gene sequence of the type strain, SH1^T. The sequences were imported to the ARB software packages (16) and aligned by ClustalW. Maximum likelihood, maximum parsimony, and neighbor joining were performed to calculate trees of single gene loci or concatenated sequences of two or nine housekeeping genes. Similarity matrices were calculated in ARB on the nucleotide level, since the resolution on the amino acid level was too low for such closely related organisms.

G+C content determination. DNA was isolated using the classical method of Marmur (18), modified by a final dialysis on a 0.025-μm-pore-size membrane (Millipore) against 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The mol% G+C of genomic DNA was determined by thermal denaturation in 0.1× SSC buffer according to the method of Marmur and Doty (19), using a DU-640 spectrophotometer (Beckman Coulter, Krefeld, Germany) equipped with a multicell changer with a thermostat and a temperature controller. Genomic DNA from *Escherichia coli* K-12 (G+C content, 51 mol%) and *Bacillus subtilis* DNA (G+C content, 43 mol%) were used as standards.

TABLE 2. DNA-DNA hybridization

Strain	% Similarity to strain[a]:				G+C content (mol%)
	SH1^T (OTU A)	CS11 (OTU B)	6C (OTU B)	CS96 (OTU C)	
SH1^T	100	79.1	77.7	54.2	54.9[b]
SH126	99.3	62.9	69.2	41.5	55.4
CS11	61	99.9	82.6	57.2	54.5
6C	55	84	100	42.1	55.2
CS7	63.8	118.3	97.7	61.4	55.6
CS8	60.8	83	95.8	43	53.9
3SC	61.2	70.3	79.5	ND	54.7
CS115	ND	74.3	81.9	50.5	ND
SM38	54.8	68.3	76.2	51.9	55.0
CS70	56.3	ND	86	42.4	55.4
2S	52.6	ND	90.3	44.7	55.2
1SC	48.7	46.2	76.7	41.2	55.6
4SC	51.5	36.1	78.3	ND	54.4
SM41	ND	24	26.4	97.9	54.5
CS96	38.1	54.9	34.9	100	55.7
SM1	38	52.8	52.7	56.9	ND
Pooled SD	0.3–0.7	0.6–1.0	0.6–1.0	0.3–0.7	0.1–1.6

[a] The DNA of these strains was labeled with DIG-11-dUTP and biotin-16-dUTP. The hybridization group is given in parentheses. ND, not determined.
[b] The G+C content of *R. baltica* SH1^T is 55.0 mol% according to Schlesner et al. (32).

DDH. DNA-DNA-hybridization was performed according to the protocol of Urdiain et al. (37).

BOX-PCR. The BOX-PCR method described by Sikorski and colleagues (33) was performed. PCR products were visualized on a 1.5% agarose gel, stained with ethidium bromide, and documented by a UV light detection system. Amplicon pictures were analyzed in the Totallab TL120 v2006e packages (Nonlinear Dynamics Inc., Newcastle upon Tyne, United Kingdom) to obtain binary matrices and to calculate trees according to the unweighted pair group method using average linkages (UPGMA).

Electron microscopy. Cells were prepared for transmission electron microscopy as described in detail elsewhere (7, 28). In brief, for visualizing whole cells, cells in suspension were chemically fixed by adding glutaraldehyde (final concentration, 2%), concentrated by brief centrifugation, applied to carbon-coated copper grids, and shadowed with 1 nm Pt/C (angle, 15°). For freeze-etching, unfixed, concentrated cells were quickly frozen in liquid nitrogen, fractured in a high-vacuum chamber (CFE 50; Cressington, Watford, England), etched for 4 min, and shadowed with 1 nm Pt/C (45°) and 10 nm C (90°). For ultrathin sections, a pellet of unfixed cells was immobilized by high-pressure freezing (Leica EM-Pact2), freeze-substituted in acetone-2% OsO_4-5% H_2O, and embedded in Epon. All electron micrographs were recorded digitally (a slow-scan charge-coupled-device [CCD] camera; TVIPS, Gauting, Germany) on a Philips CM12 transmission electron microscope operated at 120 kV.

Nucleotide sequence accession numbers. The gene sequences were deposited in GenBank under accession no. GU254991 to GU255465.

RESULTS

A collection of *R. baltica* strains from the Baltic Sea had been described as one DNA-DNA hybridization group, together with the type strain (32). The range of G+C values in the species was within the commonly reported values for a single species, 53 to 57 mol% G+C (30). For the strains SH1^T, SH155, and SH398, reported to have G+C contents of 55 mol%, we determined 54.9, 56.6, and 53.7 mol%, respectively. These values were within the uncertainty of the method. The G+C contents of 16 new isolates ranged between 53.9 and 56.5 mol% (Table 2; see Table S2 in the supplemental material), supporting the identification of the strains as members of the genus *Rhodopirellula*.

DDH has been generally used as the gold standard to circumscribe species on a genomic basis (29). Two strains are assigned to one genospecies if the DDH reveals high DNA similarity that may range between 60 to 100%. There is no absolute boundary for species circumscription, which must finally be supported by complementary phenotypic and chemotaxonomic data. Practically, a threshold of 70%, together with phenotypic and chemotaxonomic similarities, is widely accepted as the species border (29). In this work, we used the type strain, SH1^T, and strain SH126 to confirm the results of Schlesner and colleagues (32) and included 14 new isolates in the DDH experiments. Applying the protocol introduced by Urdiain and colleagues (37), we observed nearly 100% similarity of strain SH1^T with strain SH126. However, the DNA of the type strain reassociated at 38 to 63.8% with the rest of the isolates (Table 2). Additional experiments with labeled DNA from the isolated strains 6C, CS11, and CS96 revealed four genetically defined OTUs: OTU A was represented by the type strain, SH1^T, and the strain SH126. OTU B comprised strains 2S, 6C, 1SC, 3SC, 4SC, CS7, CS8, CS11, CS70, CS115, and SM38. Strain CS96 formed a hybridization group with SM41 (OTU C). Finally, strain SM1 showed low reassociation values with any labeled DNA, and thus, SM1 represented OTU D. Reciprocal values were generally comparable. Interestingly, the reference strains of OTUs B and C reassociated with 16 to 22% higher values (54 to 79%) with OTU A than the reciprocal DDH (38 to 61%) (Table 2). It is not clear why such phenomena occur, but it is most likely due to the different genome sizes. Larger genomes, e.g., *R. baltica* SH1^T with 7.15 Mbp, may show lower hybridization values with small genomes, whereas the reciprocal may show higher DDH values.

MLSA is currently under consideration as a method to potentially substitute for whole-genome hybridizations (35). *In silico* comparisons of whole genomes provided a species threshold for MLSA: related strains within one species had an average nucleotide identity in conserved genes above 94% (12). A recently published MLSA study of *Ensifer* and related taxa (20) found similar threshold values. According to metagenomic analyses, there seems to be an underrepresentation of genes with 90% to 94% identity in the environment (11). Fol-

TABLE 3. MLSA overview

OTU	Sequence identity of partial genes to *R. baltica* SH1^T (%)[b]									
	16S rRNA	*acsA*	*fumC*	*glpF*	*glyA*	*guaA*	*icd*	*mdh*	*purH*	*trpE*
R. baltica strains[a]	97.6–100	88–100	91–100	89–100	95–100	90–98	89–97	82–100	90–100	91–100
A	99.6–100	92–100	97–100	97–100	95–100	98–100	97–100 (91)[c]	98–100	94–100	94–100
B	99.5–100	87–89	90–91	89–90	89–91	90–91	89–90 (100)[d]	92–94	91–94	91–93
C	97.7–97.9	±	80 and ±[f]	89	100 and ±[f]	−	−	81–82	±	92 and ±[f]
D	96.1	−	−	90	−	−	−	76	99	96
E	95.8	99	−	99	−	−	−	78	−	87
F	98.6	−	88	99	96	−	−	96	~	93
G	96.6	−	−	−	−	−	−	−	±	−
H	95.2–96.3	89–97	76–78	88–90	−	−	−	77–81	94–100	91–93 (100)[e]
I	99.3–99.5	85	85	82–89	89–90	88	82–83	92	±	91
J	96.2–96.4	±	79 and ±[f]	89	78–79	−	−	78–80	±	−
K	96.9	76	~	−	±	−	−	−	−	~
L	94.6	±	−	88	−	−	−	−	−	−
M	93.6	±	75	88	−	−	−	−	−	−

[a] *R. baltica* strain collection from the Bay of Kiel (32).
[b] ±, amplicon was obtained but the sequence revealed another gene; −, no amplicon was obtained; ~, incomplete sequence.
[c] *icd* of strain SWK14 in OTU A had an unusual low identity of 91%.
[d] *icd* of strain 8C in OTU B had an unusual high identity of 100%.
[e] *trpE* of strains SM26 and SM32 in OTU H had unusual high identities of 100%.
[f] Values indicate that the correct amplicon was obtained from some strains within the hybridization group, but an undesired amplicon was also observed.

lowing the suggestions of Maiden and colleagues (17), we developed an MLSA with an alignment of 5,026 bp comprising a concatenate of the partial sequences of *acsA*, *guaA*, *trpE*, *purH*, *glpF*, *fumC*, *icd*, *glyA*, and *mdh*. The 16S rRNA gene sequences, the DDH experiments, and the strain geography—the 70 strains were isolated from 19 locations, with up to 12 strains originating from one 100-μl sample (40)—served as initial guidance to explore the diversity with a few MLSA primers. Based on the initial MLSA sequence similarities, we selected the most diverse strains for the complete MLSA study.

Eleven strains originating from the Baltic Sea and characterized as strains of *R. baltica* (32) were analyzed by MLSA. Ten strains had more than 99% 16S rRNA sequence identity with the type strain, with the exception of strain SH155 (97.6%). The MLSA was expected to assign all *R. baltica* strains to OTU A. Six strains associated with all partial gene sequences in the OTU A: strains SH26, SH28, SH123, SH126, SH190, and SH198. Five strains had some partial gene sequences with an identity above 94% and some with an identity below 93%. This indicated that a value of 94% similarity as a criterion for a single partial gene may not be appropriate and confirmed the necessity to analyze multiple loci. Also, strains of OTUs D, E, and F had some partial gene sequences with an identity above 94% to that of the type strain (Table 3).

The 16S rRNA gene sequences, together with the MLSA and the DDH experiments, allowed us to define 13 OTUs, OTU A to OTU M, that represent genotypes (Table 3 and Fig. 1). Of our isolates, only some strains from the west coast of Sweden and from Sylt on the German North Sea coast could be assigned to OTU A and the type strain. Thirty-one strains from our collection belonged to OTU B, which is very closely related to OTU A. Nine strains from OTU A and 18 strains from OTU B, representing all sampling sites from which strains of these OTUs were obtained, together with OTU I, were characterized by MLSA, including alignment and phylogenetic-tree construction (Table 3; see Table S1 in the supplemental material). The concatenated sequences had a length of 5,026 bp. Calculation of different trees with neighbor joining, maximum parsimony, and maximum likelihood showed a clear division between OTUs A, B, and I, comprising strains from the Baltic Sea, a region from the Netherlands to Greece, and the North Atlantic, respectively (Fig. 2). The phylogeny of the concatenation of three to seven genes yielded the same branching into three clusters (data not shown).

To confirm independently the separation of OTUs A and B, we performed a BOX-PCR analysis. The pattern of the BOX amplicons served to calculate a UPGMA tree that showed a dissimilarity of 50% between the two clusters (Fig. 3). Within OTU B, the strains from the British Channel and the North Sea (CS7, CS8, CS11, and K1070) were separated from two groups with isolates from the Mediterranean Sea. One consisted solely of strains from Porto Cesareo, Italy, and the second included strains from Porto Cesareo and Elba, both in Italy, and Piraeus, Greece.

MLSA is a taxonomically valuable tool for closely related strains, but not all species in a genus may amplify using primers derived from a single genome. We could amplify *glpF* and *mdh* from many strains with a functional sequence of the desired gene, but some other genes could be amplified from only a few strains or the amplicon had a sequence of an undesired gene (Table 3). The diversity of the strains was visualized by the reconstruction of a phylogenetic tree, using a concatenate of the sequences of the genes *glpF* and *mdh* (Fig. 1). The type strain of *R. baltica* affiliated with strains from the Bay of Kiel, the Swedish Skagerak, and the German North Sea (OTU A) and one Swedish strain, SWK 7. This isolate was placed, based on the 16S rRNA gene sequence dissimilarity, in a separate unit (OTU F). Isolates from Helgoland in the North Sea, Yerseke at the Scheldt estuary on the English Channel, and Mediterranean sites in Italy and Greece formed OTU B. The other units were represented by few isolates. The overall structure of the tree resembled the tree of the 16S rRNA gene (40). In summary, the MLSA study provided clear evidence for a taxonomic fine resolution of two 16S rRNA-defined taxonomic

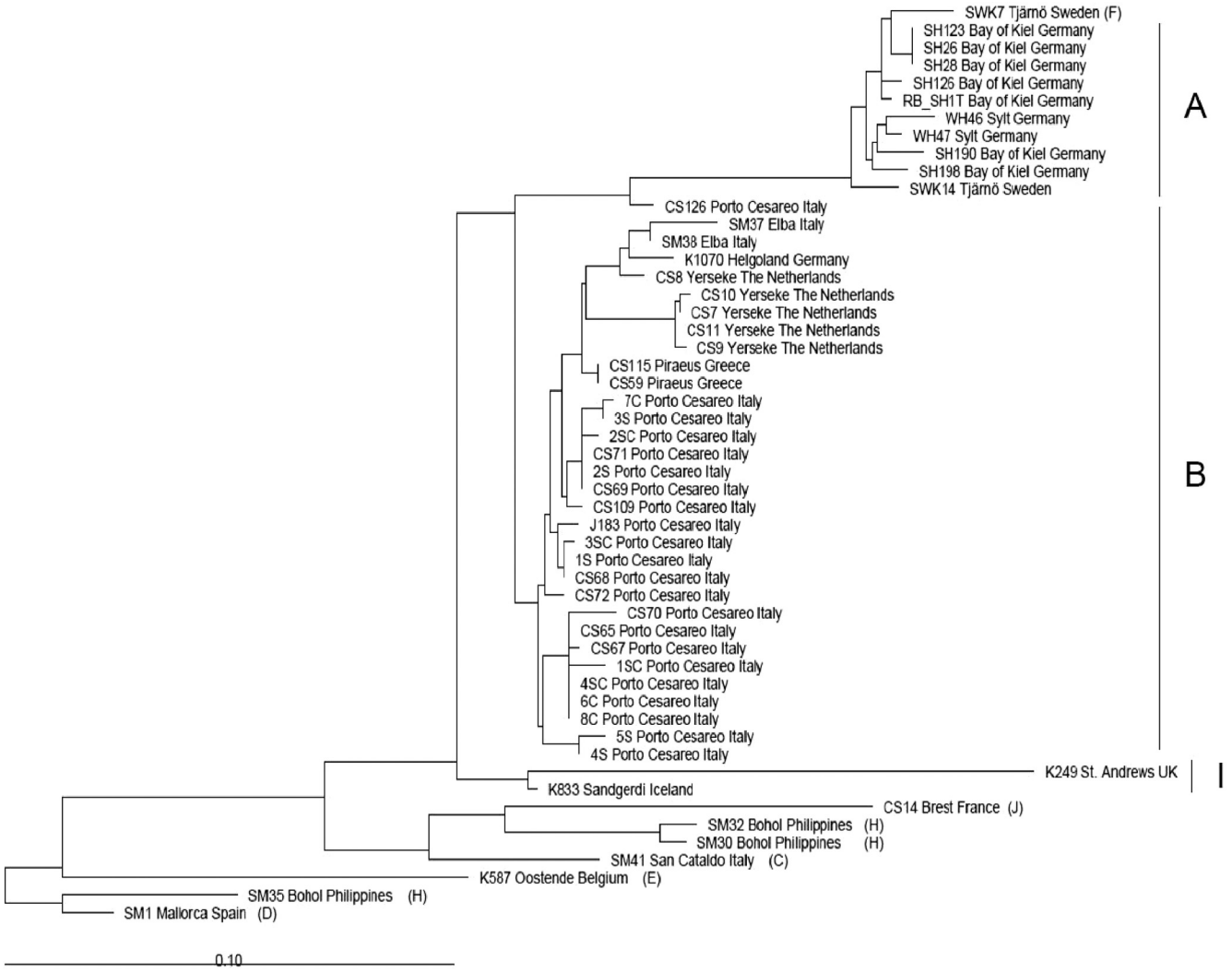

FIG. 1. Phylogenetic tree of different clusters with concatenated partial sequences of the housekeeping genes *mdh* and *glpF*, calculated in ARB with RAxML and a 50% base frequency filter. OTU groups are indicated by letters in parentheses.

units into one cluster consisting of OTUs A, B, and I and a second containing OTUs D, E, and H.

A unique feature of *Planctomycetes* is the intracellular compartmentation of the prokaryotic cells. To visualize this in our newly isolated strains and to investigate morphological differences, we employed transmission electron microscopy of cells from liquid cultures in the logarithmic growth phase. After EM preparation using three different techniques, we elucidated the structural characteristics of the type strain, SH1^T, and the isolates 6C and SM1. Cells of all three strains occurred singly, in pairs, or in aggregates. They were round to ovoid, and their average diameter was in the range of 1 to 2 μm. The type strain revealed clearly visible intracellular compartmentation in freeze-etched samples: the riboplasm was surrounded by the paryphoplasm, which contained small structures, likely vesicles or storage compartments (Fig. 4c). The image shows a small cell, likely a freshly budded cell, in contact with the large cell close to the riboplasm. Strain 6C, a member of OTU B, had a similar appearance: a large paryphoplasm with internal structures surrounded the riboplasm, and a new cell was in the budding process (Fig. 4d). Low-angle shadowing with platinum (Fig. 4a) revealed the polar structure of the cell: one pole had a smooth surface, whereas the other pole hosted thin filamentous structures. These structures were found frequently in cells prepared in this way: in addition, a physical link between the cells was evident. Further details were visible in the ultrathin-section images (Fig. 4e). The pole at the riboplasm had short, pilus-like structures and an extracellular matrix. The smooth pole had several cellular appendages in the image. This may have been due to the fixative glutaraldehyde: Lindsay et al. (14) reported a cell morphology change in the freshwater planctomycete *Gemmata oscuriglobus* upon incubation with glutaraldehyde. However, these appendages were also visible on the surface of the cell in the platinum shadowing (Fig. 4a). Appendages also covered one pole of cells of strain SM1 (Fig. 4b). The middle part of the cell had apparently collapsed due to air drying. Ultrathin sections of cells of this strain revealed an unusual intranucleosomal fibrillar structure (Fig. 4f). Such a structure was also observed in cells of *Blastopirellula marina* (13). The intracellular membrane was visible as a thin line,

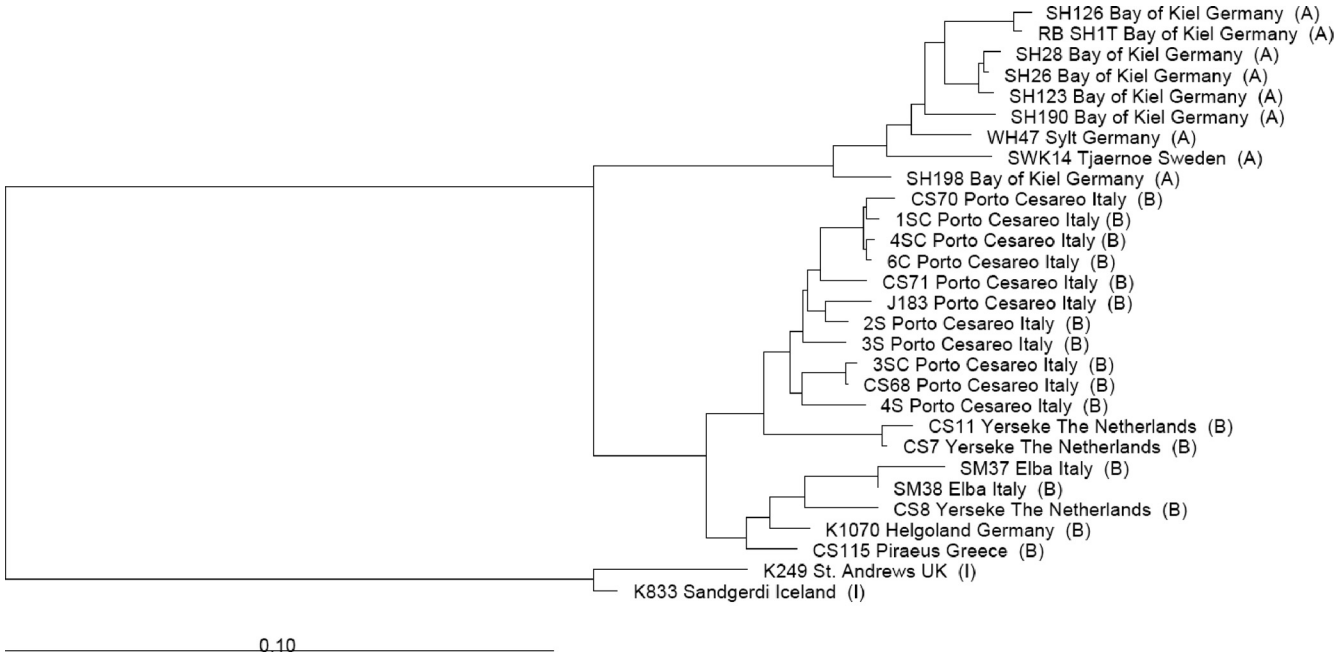

FIG. 2. Phylogenetic tree of clusters A, B, and I with concatenated partial sequences of 9 different housekeeping genes, calculated in ARB with RaxML. OTU groups are indicated by letters in parentheses.

dividing a light-gray and a dark-gray area with a black spot, the nucleoid. The outer membrane was often seen to be asymmetric: the outer leaflet was sharp, while the inner leaflet appeared "fuzzy." The micrographs supported the identification of strains 6C and SM1 as members of the *Planctomycetes*. The similarity of the images of strain 6C and the type strain may indicate close kinship, whereas the round morphology of strain SM1 suggests a distant relatedness.

DISCUSSION

The sequencing and phylogenetic reconstruction of several housekeeping genes known as MLSA has become a rapid and reliable technique to identify microbial species and strains, especially in clinical microbiology. Here, we developed and applied an MLSA to characterize strains of *Rhodopirellula* from European seas. For strains with 16S rRNA gene similarity of less than 99.0% to *R. baltica* SH1^T, the experiments indicated a low applicability of the designed MLSA primers. Only the amplicons for the glycerol facilitator uptake protein (*glpF*) and malate dehydrogenase (*mdh*) were useful for distantly related strains in the genus. Other studies have successfully applied MLSA to *Ensifer*, *Pseudomonas*, and *Vibrio* species (3, 21, 36), not only for differentiation on the intraspecies level, but also between several distantly related species. The development of such a genus-wide MLSA scheme for *Rhodopirellula* can make use of the *glpF* and *mdh* primer pairs, but additional genomic information from several strains is needed to develop a *Rhodopirellula* genus-wide MLSA. Previous studies, e.g., of *Vibrio* (36), had established a clear correlation between the results of DDH studies and those of MLSA studies, and it has been suggested to replace the former technique with the latter. We still tested our MLSA results with DDH experiments and additionally applied a BOX-PCR for high taxonomic resolution. Electron microscopy provided morphological evidence for the identification as *Planctomycetes* and revealed differences between the OTUs. Together with the 16S rRNA phylogeny (40), we obtained a congruent picture of the genetic diversity in the strain collection.

MLSA and MLST studies identify genetic identities and provide insight into the biogeography and epidemiology of microorganisms. In the genetically designated PVC superphylum of *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae* (38), MLST approaches were applied to reveal the history of pathogenic *Chlamydiae* strains (10, 26, 42). *Vibrio*, *Pseudomonas*, and *Salinibacter* are marine microorganisms that were recently studied with MLSA. Thompson and colleagues (36) reported a clear separation of *Vibrio* species by MLSA and confirmed these results by DNA-DNA hybridization and 16S rRNA phylogeny. The MLST study of Khan and colleagues (8) identified distinct clusters of oceanic strains inside the species *Pseudomonas aeruginosa*, reflecting the geographic locations of the isolation sites. An MLSA study of the extremophilic bacterium *Salinibacter ruber* (31) did not detect a biogeography among the isolates from the Mediterrean Sea, the Atlantic Ocean, and the Peruvian upwelling region. The application of high-resolution mass spectrometry (Fourier transform ion cyclotron resonance mass spectrometry) revealed a fine-scale diversity in association with geographical patterns. This result revealed a resolution border of MLSA based on the housekeeping genes selected for the MLSA approach. A biogeography may also be found in the secondary metabolism or on regulatory levels, in accordance with environmental conditions. Conversely, a study of *Sulfolobus* isolates from hot acidic and neutral lakes in Kamchatka, East Africa, and North America found a biogeography among the isolates with an MLSA approach (39). We

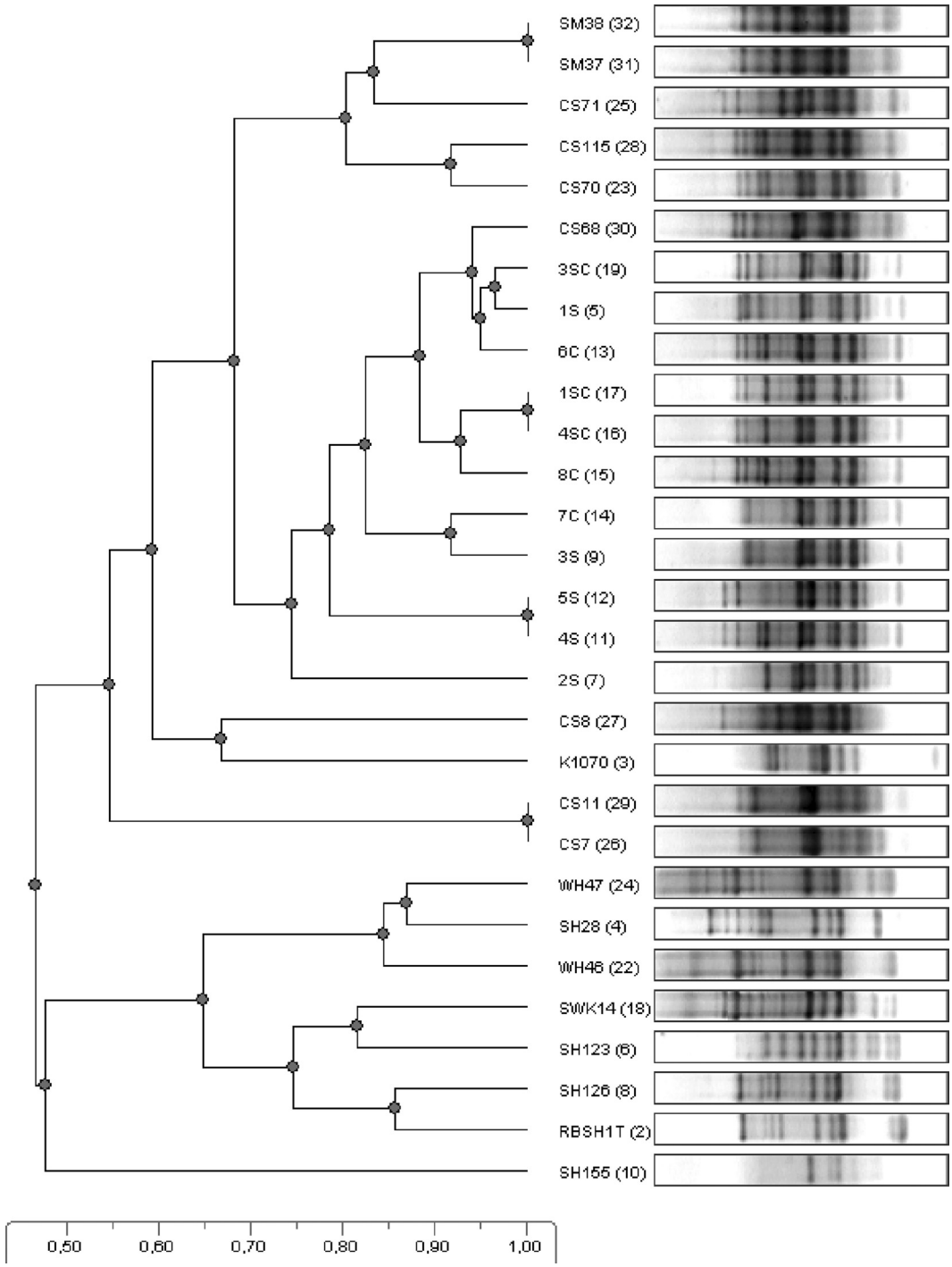

FIG. 3. BOX-PCR analysis of strains affiliated with OTUs A and B. The patterns were obtained by gel electrophoreses and analyzed with Totallab TL120 software (Nonlinear Dynamics Inc., Newcastle upon Tyne, United Kingdom). The numbers in parentheses show the original lane numbers in the gel. Missing lane numbers are due to marker lanes or insufficient resolution of band patterns.

hypothesized that an attached-living bacterium may be hampered in its dispersal and, hence, a biogeography may be detectable by MLSA. So far, biogeographical studies have focused only on free-living microorganisms from various environments.

The genetic analysis of *Rhodopirellula* spp. showed evidence for a biogeography in European seas. Isolates of OTU A, including *R. baltica* strains from the Bay of Kiel, were restricted to parts of the Baltic Sea, Skagerrak, and the east coast of the North Sea. Strains of OTU B were isolated from the southern North Sea to the Mediterranean Sea. Isolates from Scotland and Iceland indicated a habitat in the North Atlantic (OTU I).

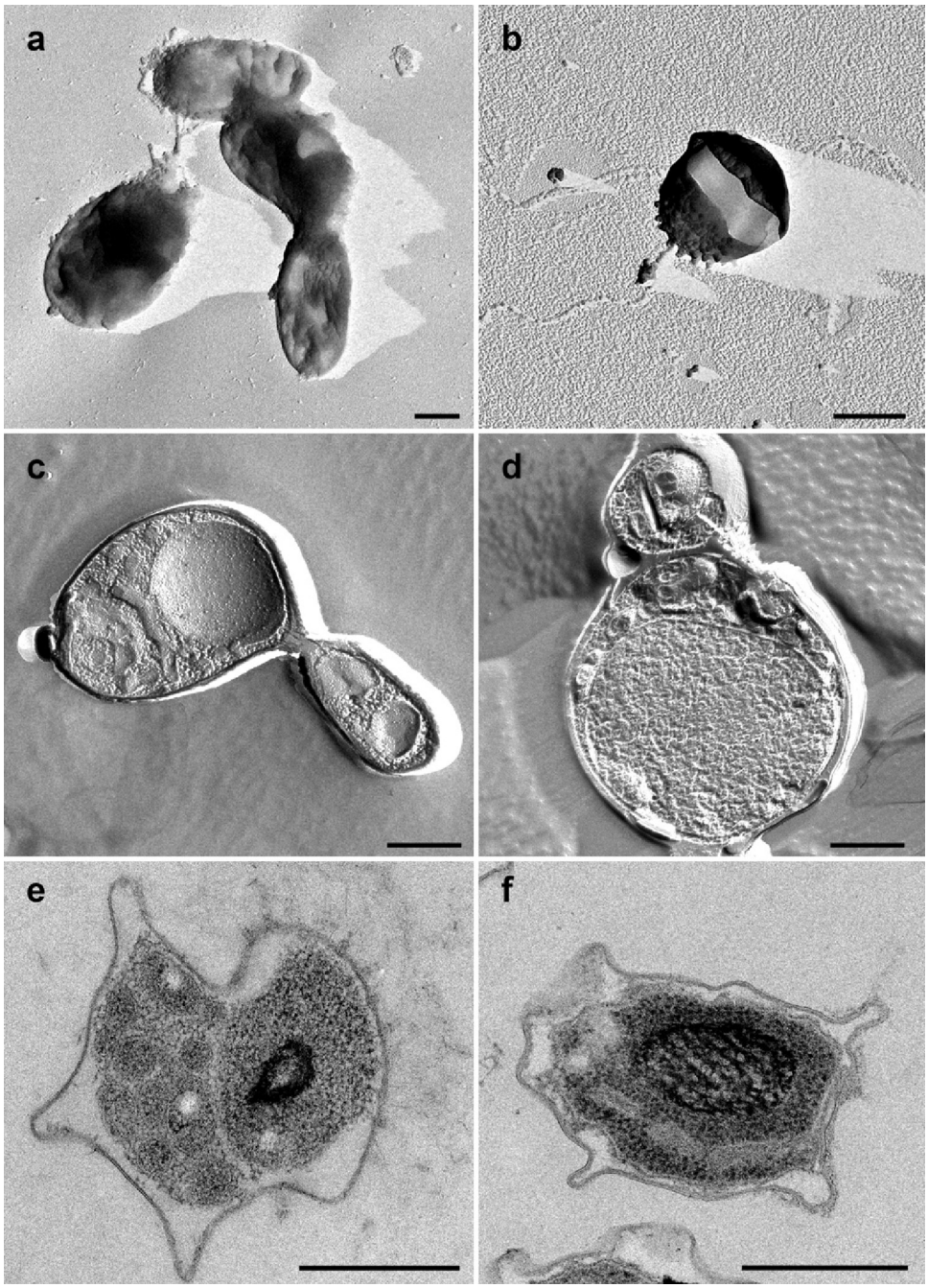

FIG. 4. Micrographs of *R. baltica* SH1^T (c), *Rhodopirellula* sp. strain 6C (a, d, and e), and strain SM1 (b and f). Images a and b show Pt-shadowed cells, c and d show freeze-etch preparations, and e and f show ultrathin sections of embedded cells. Bars, 0.5 μm.

BOX-PCR revealed clustering according to regions within OTU B, separating the isolates from the North Sea and the British Channel from the Mediterranean isolates. The other OTUs were present with a low number of isolates in the strain collection. Therefore, their habitat sizes cannot be predicted. Besides the restriction of the habitat to a region, a niche differentiation may explain the observations. In the future, the cultivation-based studies have to be complemented by physiological studies of the strains and by cultivation-independent experiments with habitat samples to reveal the influence of the environment on the habitat size.

We detected 13 OTUs in 70 strains from 19 sample sites. The cultivated genetic diversity, together with the presence of genes closely related to *R. baltica* in different habitats all over the world (23, 24, 25, 27, 41), is in agreement with a recent ecological hypothesis of Kirchman and colleagues (9): bacterial groups in aquatic systems that appear to be more widespread are more diverse. Theoretically, biogeographical distributions

may have evolved from environmental variations or historical events (8). Without a large data set of *Planctomycetes* strain diversity and consequently a gene-based tree of the development, we can only highlight some environmental factors. Temperatures at the sample sites varied between 5.2°C in the North Sea and 21.5°C in the Mediterranean Sea, while non-European isolates were obtained from sites at 26.3°C in Asia or 28.9°C off the coast of Africa (40). These warm-water strains formed OTUs H and K and differed from European strains. Salinity values varied from 21 to 38.2 practical salinity units, which is in the range of salinity tolerance published for *R. baltica* SH1^T.

A large number of taxon-area relationships are often achieved by cultivation-independent methods. However, genetic-fingerprinting methods, e.g., denaturing gradient gel electrophoresis (DGGE), automated ribosomal intergenic spacer analysis (ARISA), and terminal restriction fragment length polymorphism (T-RFLP), as well as 16S rRNA gene clone libraries, have low taxonomic resolution above the species level. Sequences of housekeeping genes can provide the desired genetic resolution on the species level. The MLSA characterization of *Rhodopirellula* isolates has revealed a broad genetic diversity in European seas and has provided the first evidence for a limited habitat size of attached-living bacteria. In the future, cultivation-independent studies within the genus applying the *glpF* and *mdh* primers will allow more precise determinations of the habitat size.

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REFERENCES

1. **Aanensen, D. M., and B. G. Spratt.** 2005. The multilocus sequence typing network: mlst.net. Nucleic Acids Res. **33:**W728–W733.
2. **Achtman, M.** 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu. Rev. Microbiol. **62:**53–70.
3. **Cladera, A. M., L. D. C. Sepulveda-Torres, M. Valens-Vadell, J. M. Meyer, J. Lalucat, and E. Garcia-Valdes.** 2006. A detailed phenotypic and genotypic description of *Pseudomonas* strain OX1. Syst. Appl. Microbiol. **29:**422–430.
4. **Gade, D., H. Schlesner, F. O. Glockner, R. Amann, S. Pfeiffer, and A. Thomm.** 2004. Identification of *Planctomycetes* with order-, genus-, and strain-specific 16S rRNA-targeted probes. Microb. Ecol. **47:**243–251.
5. **Glöckner, F. O., M. Kube, M. Bauer, H. Teeling, T. Lombardot, W. Ludwig, D. Gade, A. Beck, K. Borzym, K. Heitmann, R. Rabus, H. Schlesner, R. Amann, and R. Reinhardt.** 2003. Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc. Natl. Acad. Sci. U. S. A. **100:**8298–8303.
6. **Ivars-Martinez, E., G. D'Auria, F. Rodriguez-Valera, C. Sanchez-Porro, A. Ventosa, I. Joint, and M. Muhling.** 2008. Biogeography of the ubiquitous marine bacterium *Alteromonas macleodii* determined by multilocus sequence analysis. Mol. Ecol. **17:**4092–4106.
7. **Junglas, B., A. Briegel, T. Burghardt, P. Walther, R. Wirth, H. Huber, and R. Rachel.** 2008. *Ignicoccus hospitalis* and *Nanoarchaeum equitans*: ultrastructure, cell-cell interaction, and 3D reconstruction from serial sections of freeze-substituted cells and by electron cryotomography. Arch. Microbiol. **190:**395–408.
8. **Khan, N. H., M. Ahsan, S. Yoshizawa, S. Hosoya, A. Yokota, and K. Kogure.** 2008. Multilocus sequence typing and phylogenetic analyses of *Pseudomonas aeruginosa* isolates from the ocean. Appl. Environ. Microbiol. **74:**6194–6205.
9. **Kirchman, D. L., A. I. Dittel, R. R. Malmstrom, and M. T. Cottrell.** 2005. Biogeography of major bacterial groups in the Delaware Estuary. Limnol. Oceanogr. **50:**1697–1706.
10. **Klint, M., H. H. Fuxelius, R. R. Goldkuhl, H. Skarin, C. Rutemark, S. G. E. Andersson, K. Persson, and B. Herrmann.** 2007. High-resolution genotyping of *Chlamydia trachomatis* strains by multilocus sequence analysis. J. Clin. Microbiol. **45:**1410–1414.
11. **Konstantinidis, K. T., and E. F. DeLong.** 2008. Genomic patterns of recombination, clonal divergence and environment in marine microbial populations. ISME J. **2:**1052–1065.
12. **Konstantinidis, K. T., and J. M. Tiedje.** 2005. Towards a genome-based taxonomy for prokaryotes. J. Bacteriol. **187:**6258–6264.
13. **Lindsay, M. R., R. I. Webb, and J. A. Fuerst.** 1997. Pirellulosomes—a new type of membrane-bounded cell compartment in planctomycete bacteria of the genus *Pirellula*. Microbiology **143:**739–748.
14. **Lindsay, M. R., R. I. Webb, H. M. Hosmer, and J. A. Fuerst.** 1995. Effects of fixative and buffer on morphology and ultrastructure of a freshwater planctomycete, *Gemmata obscuriglobus*. J. Microbiol. Methods **21:**45–54.
15. **Louws, F. J., D. W. Fulbright, C. T. Stephens, and F. J. Debruijn.** 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. **60:**2286–2295.
16. **Ludwig, W., S. O. Westram, R. Richter, L. Meier, H. Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
17. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing—a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. **95:**3140–3145.
18. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
19. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **4:**93–103.
20. **Martens, M., M. Delaere, R. Coopman, P. De Vos, M. Gillis, and A. Willems.** 2007. Multilocus sequence analysis of *Ensifer* and related taxa. Int. J. Syst. Evol. Microbiol. **57:**489–503.
21. **Martens, M., P. Dawyndt, R. Coopman, M. Gillis, P. De Vos, and A. Willems.** 2008. Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). Int. J. Syst. Evol. Microbiol. **58:**200–214.
22. **Martiny, J. B. H., B. J. M. Bohannan, J. H. Brown, R. K. Colwell, J. A. Fuhrman, J. L. Green, M. C. Horner-Devine, M. Kane, J. A. Krumins, C. R. Kuske, P. J. Morin, S. Naeem, L. Ovreas, A. L. Reysenbach, V. H. Smith, and J. T. Staley.** 2006. Microbial biogeography: putting microorganisms on the map. Nat. Rev. Microbiol. **4:**102–112.
23. **Morris, R. M., K. Longnecker, and S. J. Giovannoni.** 2006. *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. Environ. Microbiol. **8:**1361–1370.
24. **Musat, N., U. Werner, K. Knittel, S. Kolb, T. Dodenhof, J. E. E. van Beusekom, D. de Beer, N. Dubilier, and R. Amann.** 2006. Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Romo Basin, Wadden Sea. Syst. Appl. Microbiol. **29:**333–348.
25. **Neef, A., R. Amann, H. Schlesner, and K. H. Schleifer.** 1998. Monitoring a widespread bacterial group: in situ detection of *Planctomycetes* with 16S rRNA-targeted probes. Microbiology **144:**3257–3266.
26. **Pedersen, L. N., B. Herrmann, and J. K. Moller.** 2009. Typing *Chlamydia trachomatis*: from egg yolk to nanotechnology. FEMS Immunol. Med. Microbiol. **55:**120–130.
27. **Pimentel-Elardo, S., M. Wehrl, A. B. Friedrich, P. R. Jensen, and U. Hentschel.** 2003. Isolation of planctomycetes from *Aplysina* sponges. Aquat. Microb. Ecol. **33:**239–245.
28. **Rachel, R., I. Wyschkony, S. Riehl, and H. Huber.** 2002. The ultrastructure of *Ignicoccus*: evidence for a novel outer membrane and for intracellular vesicle budding in an archaeon. Archaea **1:**9–18.
29. **Rosselló-Mora, R.** 2006. DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation, p. 23–50. *In* E. Stackebrandt (ed.), Molecular identification, systematics, and population structure of prokaryotes. Springer, Heidelberg, Germany.
30. **Rosselló-Mora, R., and R. Amann.** 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. **25:**39–67.
31. **Rosselló-Mora, R., M. Lucio, A. Pena, J. Brito-Echeverria, A. Lopez-Lopez, M. Valens-Vadell, M. Frommberger, J. Anton, and P. Schmitt-Kopplin.** 2008. Metabolic evidence for biogeographic isolation of the extremophilic bacterium *Salinibacter ruber*. ISME J. **2:**242–253.
32. **Schlesner, H., C. Rensmann, B. J. Tindall, D. Gade, R. Rabus, S. Pfeiffer, and P. Hirsch.** 2004. Taxonomic heterogeneity within the *Planctomycetales* as derived by DNA-DNA hybridization, description of *Rhodopirellula baltica* gen. nov., sp nov., transfer of *Pirellula marina* to the genus *Blastopirellula* gen. nov. as *Blastopirellula marina* comb. nov and emended description of the genus *Pirellula*. Int. J. Syst. Evol. Microbiol. **54:**1567–1580.
33. **Sikorski, J., R. Rosselló-Mora, and M. G. Lorenz.** 1999. Analysis of genotypic diversity and relationships among *Pseudomonas stutzeri* strains by PCR-

based genomic fingerprinting and multilocus enzyme electrophoresis. Syst. Appl. Microbiol. **22:**393–402.
34. **Stackebrandt, E., and J. Ebers.** 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today **33:**152–155.
35. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimont, P. Kampfer, M. C. J. Maiden, X. Nesme, R. Rosselló-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. **52:**1043–1047.
36. **Thompson, F. L., B. Gomez-Gil, A. T. R. Vasconcelos, and T. Sawabe.** 2007. Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. Appl. Environ. Microbiol. **73:**4279–4285.
37. **Urdiain, M., A. López-López, C. Gonzalo, H.-J. Busse, S. Langer, P. Kämpfer, and R. Rosselló-Mora.** 2008. Reclassification of *Rhodobium marinum* and *Rhodobium pfennigii* as *Afifella marina* gen. nov. comb. nov. and *Afifella pfennigii* comb. nov., a novel genus of photoheterotrophic Alphaproteobacteria and emended descriptions of *Rhodobium*, *Rhodobium orientis* and *Rhodobium gokarnense*. Syst. Appl. Microbiol. **31:**339–351.
38. **Wagner, M., and M. Horn.** 2006. The *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr. Opin. Biotechnol. **17:**241–249.
39. **Whitaker, R. J., D. W. Grogan, and J. W. Taylor.** 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science **301:**976–978.
40. **Winkelmann, N., and J. Harder.** 2009. An improved isolation method for attached-living planctomycetes of the genus Rhodopirellula. J. Microbiol. Methods **77:**276–284.
41. **Woebken, D., H. Teeling, P. Wecker, A. Dumitriu, I. Kostadinov, E. F. DeLong, R. Amann, and F. O. Glöckner.** 2007. From the Namibian and Oregon coast upwelling systems and their cross-comparison with planctomycete genomes. ISME J. **1:**419–435.
42. **Yousef Mohamad, K., S. M. Roche, G. Myers, P. M. Bavoil, K. Laroucau, S. Magnino, S. Laurent, D. Rasschaert, and A. Rodolakis.** 2008. Preliminary phylogenetic identification of virulent *Chlamydophila pecorum* strains. Infect. Genet. Evol. **8:**764–771.