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Real-Time Quantitative PCR for Assessment of Abundance of *Pseudoalteromonas* Species in Marine Samples

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A real-time quantitative PCR (RTQ-PCR) method for measuring the abundance of *Pseudoalteromonas* species in marine samples is presented. PCR primers targeting a *Pseudoalteromonas*-specific region of the 16S rRNA gene were tested at three different levels using database searches (in silico), a selection of pure cultures (in vitro), and a combined denaturing gradient gel electrophoresis and cloning approach on environmental DNA (in situ). The RTQ-PCR method allowed for the detection of SYBR Green fluorescence from double-stranded DNA over a linear range spanning six orders of magnitude. The detection limit was determined as 1.4 fg of target DNA (1,000 gene copies) measured in the presence of 20 ng of nontarget DNA from salmon testes. In this study, we discuss the importance of robust post-PCR analyses to overcome pitfalls in RTQ-PCR when samples from different complex marine habitats are analyzed and compared on a nonroutine basis. Representatives of the genus *Pseudoalteromonas* were detected in samples from all investigated habitats, suggesting a widespread distribution of this genus across many marine habitats (e.g., seawater, rocks, macroalgae, and marine animals). Three sample types were analyzed by RTQ-PCR to determine the relative abundance of *Pseudoalteromonas* ribosomal DNA (rDNA) compared to the total abundance of eubacterial rDNA. The rDNA fractions of *Pseudoalteromonas* compared to all *Eubacteria* were 1.55% on the green alga *Ulva lactuca*, 0.10% on the tunicate *Ciona intestinalis*, and 0.06% on the green alga *Ulvaria fusca*.

The marine bacterial genus *Pseudoalteromonas* contains numerous species. These species have attracted significant interest for two reasons. First, a large proportion of the *Pseudoalteromonas* species are frequently found in association with marine eukaryotic hosts such as fish, molluscs, tunicates, sponges, and micro- and macroalgae (2, 15, 24, 31, 37). Second, many of the *Pseudoalteromonas* species produce biologically active compounds, which target a broad range of marine fouling organisms (21). These bioactive compounds exhibit antibacterial, algicidal, antifungal, agarolytic, and antiviral activities (23).

A well-studied member of the genus *Pseudoalteromonas* is the green-pigmented bacterium *Pseudoalteromonas tunicata* (22). This bacterium produces at least five different extracellular compounds that specifically inhibit the growth and settlement of different classes of marine organisms (9, 10, 23, 24, 26). *P. tunicata* has been isolated from two eukaryotic hosts, the tunicate *Ciona intestinalis* and the green alga *Ulva lactuca* (11, 24). *P. tunicata* has also been isolated from an algal bloom in Tasmania, Australia, and its 16S ribosomal DNA (rDNA) sequences have been found in clone libraries sampled from biofilms in saline water caves in Australia and Antarctic sea ice (8, 20). To further evaluate the ecological significance of this bacterium and other members of the genus *Pseudoalteromonas*, we need to apply robust quantitative molecular methods with a resolution that can detect changes in the abundance of this genus.

Real-time quantitative PCR (RTQ-PCR) has been shown to be a powerful tool for quantifying bacterial abundance in many different kinds of complex environmental samples down to the genus and species levels (3, 17, 44, 48). In recent studies, the abundance of a genus or species in an environmental sample has also been related to the overall eubacterial abundance in the analyzed system (1, 5, 32, 42). RTQ-PCR provides information on the relative and absolute abundance of a genus or species in complex environmental samples. Such detailed information can often be difficult to obtain and investigate with conventional cell enumeration methods such as most probable number, CFU, and direct cell counting methods. The culture-independent fluorescence in situ hybridization (FISH) method might be an alternative approach to enumerate phylogenetically distinct bacterial populations in complex environmental samples. However, target bacteria living in biofilms on highly pigmented surfaces (e.g., macroalgae) in low numbers may be difficult to enumerate with FISH. This difficulty is due to both the high background fluorescence from algal pigments and the potentially low number of 16S rRNA target molecules per cell. Finally, the rare abundance of the phylogenetic group of interest, which in our case is at the genus level, may hinder analysis (6, 12).

Extensive evaluation of the applied taxon-specific oligonucleotides is a critical step in any quantification attempt using molecular methods such as FISH and RTQ-PCR. A substantial number of newly developed primers and probes up to the domain level have been tested only in simplified model systems

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TABLE 1. Primers used in this study

Primer	Target	Application(s)	Sequence (5′–3′)	Reference
Eub341F[a]	*Eubacteria* (16S rDNA)	RTQ-PCR and DGGE	CCTACGGGAGGCAGCAG	35
Psalt815R	*Pseudoalteromonas* (16S rDNA)	RTQ-PCR and DGGE	CCAGCTTCTAGTAGACATCGTT	20
Univ907RC	Universal (16S rDNA)	RTQ-PCR	CCGTCAATTCCTTTGAGTTT	41
26F[b]	*Eubacteria* (16S rDNA)	Standard curve	AGAGTTTGATCCTGGCTCA	19
1390R[c]	Universal (16S rDNA)	Standard curve	GACGGGCGGTGTGTACAA	50

[a] The DGGE primer had a GC clamp attached to its 5′ end: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′ (this study).
[b] 26F was formerly known as the probe EUB008.
[c] 1390R was formerly known as the probe Univ-1390-a-A-18.

with a limited number of pure cultures (33, 36). It has therefore been suggested by Schramm et al. (43) that the specificity of newly developed FISH probes be tested with a clone-FISH approach. In this method, probes are tested against clones containing environmental DNA of known origin and identity and not only against a limited number of selected pure cultures. A similar specificity test should be performed for any newly developed PCR primer by cloning and sequencing isolated nucleic acids from the environment of interest (4, 13, 38).

In this study, we have incorporated three levels of testing for the genus-specific *Pseudoalteromonas* primer Psalt815R (Table 1): (i) in silico tests using sequence databases and primer testing tools, (ii) PCR in vitro testing of a selection of closely related pure cultures, and (iii) in situ testing of nucleic acids isolated from four different environmental samples with a genus-specific PCR-DGGE (denaturing gradient gel electrophoresis) and cloning approach.

In this paper, we present a *Pseudoalteromonas*-specific RTQ-PCR protocol developed to determine the relative and absolute abundance of *Pseudoalteromonas* species in environmental samples without culturing. To our knowledge, this is the first study where RTQ-PCR has been applied to complex bacterial communities living in association with higher marine organisms. The RTQ-PCR method was applied on three different environmental sample types: *Ulvaria fusca*, *Ulva lactuca*, and *C. intestinalis*. We also show the importance of extensive primer evaluation with tests at three different levels (in silico, in vitro, and in situ) and the use of post-PCR analyses to ensure optimal abundance estimates with RTQ-PCR. Such robust quantitative information obtained with the RTQ-PCR method is vital in order to gain a better understanding of the ecological importance of the genus *Pseudoalteromonas* in the marine environment.

MATERIALS AND METHODS

Verification of primer specificity with databases, pure cultures, and environmental samples. The *Pseudoalteromonas*-specific primer Psalt815R (Table 1) was first tested by using the latest version of the probe match function at the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu/html/), the BLAST search function at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and the probe match tool in ARB (http://www.arb-home.de) together with the latest updated ARB database (June 2002).

Twenty different pure cultures were then used to test the specificity of the *Pseudoalteromonas*-specific primer set Eub341F-Psalt815R in PCR (Table 1). The *Pseudoalteromonas* species tested were *Pseudoalteromonas aurantia* (NCIMB 2033), *P. citrea* (NCIMB 1889), *P. piscicida* (NCIMB 645), *P. rubra* (NCIMB 1890), *P. undina* (NCIMB 2128), *P. ulvae* (from *Ulva lactuca*), *P. haloplanktis* (NCIMB 1545), *P. luteoviolacea* (NCIMB 1893), *P. tunicata* (from *C. intestinalis*), *P. nigrifaciens* (NCIMB 8614), *Pseudoalteromonas* sp. strain R11 (from a red alga collected in waters off the coast of Aarhus, Denmark), *Pseudoalteromonas* sp. strain x3 (from squid), and *Pseudoalteromonas* sp. strain ACEM-4 (from Tasmanian algal bloom). The other species tested were *Alteromonas maclodii* strain T52 (from the red alga *Delisea pulchra* collected in waters off the cost of Sydney, Australia), *Colwellia* sp. strain ACAM 605 (from Tasmanian seawater), *Shewanella putrefaciens* (from fish), *Vibrio campbellii* (from a marine sponge), *Pseudomonas stutzeri* (from soil), an *Agrobacterium* sp. (from soil), and *Photobacterium* sp. strain R22II (from a red alga collected in waters off the coast of Aarhus, Denmark).

The primers were also evaluated in a *Pseudoalteromonas*-specific PCR-DGGE analysis including a total of 11 different marine samples collected in December 2000 in coastal waters around Sydney, Australia. Selected DGGE bands from samples with low *Pseudoalteromonas* diversity (*C. intestinalis* and *Ulva lactuca*) were sequenced. The primer specificities were tested through cloning and sequencing by use of samples with high *Pseudoalteromonas* diversity (a *Stylinos* sp. and seawater). The following macroalgae were collected for PCR-DGGE: a *Corallina* sp., *Dictyota dichotoma*, *Ecklonia radiata*, *Ulva lactuca*, and *Codium fragile*. Collected marine animals were the sponges of a *Stylinos* sp. and *Cymbastela concentrica* and an unidentified fouled ascidian. Seawater and rocks were also collected. Individuals of the tunicate *C. intestinalis* were collected in July 2001 in Aarhus Bay, Denmark. Each environmental sample type was collected in duplicate. Sample collection and processing followed the sampling in Danish coastal waters as described below.

Collection and processing of marine samples. Three different higher marine organisms were collected in August 2002 for a detailed analysis of the abundance of *Pseudoalteromonas* species on their surfaces: *Ulvaria fusca* (Mariager Fjord), *Ulva lactuca* (Aarhus Bay), and *C. intestinalis* (Aarhus Harbor). Four individuals of each sample type were collected, extracted, and tested individually. Each individual was transferred back to the lab within 1 h in plastic bags containing seawater from the sampling site to keep the samples at in situ temperature during transportation. Seawater was removed from the plastic bags and replaced with a sterile nine-salts solution (34). Samples were rinsed three times in sterile nine-salts solution to remove nonattached bacteria from the sample surface. The cutting of samples was performed with sterile tools. Sample area, dry weight, and wet weight were measured. Approximately 1 to 10 mg of tissue (dry weight) was sampled from each individual. Samples were kept at −80°C until DNA extraction.

DNA extraction from environmental samples and pure cultures. Nucleic acids from environmental samples were extracted with the FastDNA spin kit for soil, and nucleic acids from pure cultures were extracted with the FastDNA kit (both from Qbiogene, Carlsbad, Calif.) according to the manufacturer's instructions. The method involves a mechanical cell lysis by bead beating (FastPrep DNA extractor; BIO101, Vista, Calif.) followed by selective DNA adsorption to microporous silicate filters. The DNA was washed with ethanol in the presence of chaotropic salts and finally eluted in a low-salt buffer. The nucleic acid extraction was evaluated on a 1% Seakem GTG agarose gel (FMC BioProducts, Rockland, Maine) run in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8.3]). The gel was stained with 100 ng of SYBR Green (Molecular Probes, Leiden, The Netherlands) per ml for 45 min. For analysis and documentation of DNA, a UV transilluminator and a digital camera (Gel Doc 2000; Bio-Rad, Munich, Germany) were used. Images were acquired with the Quantity One software from Bio-Rad. Raw DNA extracts used for primer evaluation were diluted 1:10 in molecular biology-grade water (Sigma, Copenhagen, Denmark) prior to DNA amplification to avoid high levels of PCR inhibitors in the DNA extract. DNA extracts used in RTQ-PCR were diluted to a final concentration of 2.5 ng/μl prior to PCR.

RTQ-PCR. The LightCycler system (Roche Molecular Biochemicals, Indianapolis, Ind.) was used with two different amplification protocols to enumerate 16S rRNA genes. The protocols were designed to enumerate 16S rRNA genes at

TABLE 2. Key measures for RTQ-PCR standard curves

Sample	PCR target	PCR efficiency (E)[a]	Mean error[b]	R value[c]
Ulvaria fusca	*Pseudoalteromonas*	1.85 ± 0.07	0.10	1.00
Ulvaria fusca	*Eubacteria*	1.81 ± 0.00	0.17	1.00
Ulva lactuca	*Pseudoalteromonas*	1.83 ± 0.02	0.08	1.00
Ulva lactuca	*Eubacteria*	1.80 ± 0.03	0.14	1.00
C. intestinalis	*Pseudoalteromonas*	1.83 ± 0.02	0.16	1.00
C. intestinalis	*Eubacteria*	1.78 ± 0.00	0.08	1.00

[a] PCR amplification efficiency $E = 10^{-1/\text{slope}}$, 100% where E_{max} equals 2. Mean of two RTQ-PCR runs ± 95% confidence interval. Total variation between all 12 runs was <2% relative standard deviation.

[b] Mean squared error, $\Sigma(\Delta x^*2)/n$, where Δx is the vertical distance between the data point and the regression line; $n = 4$ data points. The value gives a measure of tube-to-tube variation.

[c] Regression coefficient for best linear fit of the standard curve.

(i) the genus level of *Pseudoalteromonas* and (ii) the domain level of *Eubacteria* with the use of the primers shown in Table 1.

The *Pseudoalteromonas* amplification mixture contained 13.6 μl of distilled H_2O (dH_2O) (FastStart DNA Master SYBR Green I kit; Roche), 2.0 μl of 25 mM $MgCl_2$ (Roche), 0.2 μl of a 50-pmol/μl Eub341F and Psalt815R solution (MWG Biotech), and 2.0 μl of FastStart DNA Master SYBR Green I (Roche).

The *Eubacteria* amplification mixture contained 13.2 μl of dH_2O (Roche), 2.4 μl of 25 mM $MgCl_2$ (Roche), 0.2 μl of a 50-pmol/μl Eub341F and Univ907RC solution (MWG Biotech), and 2.0 μl of FastStart DNA Master SYBR Green I (Roche). The amplification mixture was loaded into precooled LightCycler glass capillary tubes followed by 2 μl of template DNA (all 2.5 ng/μl) to a final volume of 20 μl. The following RTQ-PCR program was performed on the LightCycler: an initial denaturation step at 95°C for 10 min to activate the *Taq* DNA polymerase followed by 40 (for *Pseudoalteromonas*) or 30 (for *Eubacteria*) cycles consisting of heating at 20°C/s to 95°C with a 10-s hold, cooling at 20°C/s to 57°C (for *Eubacteria*) or 62°C (for *Pseudoalteromonas*) with a 10-s hold, heating at 20°C/s to 72°C with a 20-s hold, and heating at 20°C/s to 84°C with a 6-s hold. At the end of each cycle (at 84°C), the fluorescent signal was measured at 530 nm. The RTQ-PCR program was followed by a melting curve analysis to determine the melting point of the double-stranded DNA (dsDNA) products produced. This analysis was able to verify whether amplified products had a melting profile similar to that of our target (*Pseudoalteromonas* or *Eubacteria*). The melting program consisted of continuous heating from 35 to 95°C at 0.1°C/s. Finally, the PCR products were cooled down to 40°C at 20°C/s. As an additional post-PCR analysis, all PCR products were analyzed for unspecific PCR amplification. For this purpose, diluted (1:20) amplification products (5 μl) were loaded on a 2% Nusieve 3:1 agarose gel (FMC Bioproducts) and run and analyzed as described above. Duplicate runs were performed on the LightCycler for each RTQ-PCR setup. One setup consisted of one taxonomic level and one sample type.

RTQ-PCR standard curves and assay detection limit. DNA from *P. tunicata* was used to establish a standard curve that was included in each LightCycler run. The standards contained amplified 16S rDNA from *P. tunicata* in different quantities. The primers 26F and 1390R were used for this amplification (Table 1). For the *Pseudoalteromonas*-specific protocol, a range from 6×10^3 to 2×10^8 16S rDNA gene copies/PCR setup was used, and for the *Eubacteria*-specific protocol, a range from 2×10^5 to 2×10^8 16S rDNA gene copies/PCR setup was used. Concentrations of the standards were measured fluorometrically (TD-700 fluorometer; Turner Design, Sunnyvale, Calif.). An analysis of key LightCycler measures was performed after each run to verify identical amplification efficiencies and conditions between runs (Table 2). When using this kind of standard curve, we assume equal level of amplification efficiency for 16S rDNA in *P. tunicata* and all the analyzed 16S rRNA genes present in our samples. Raw data from the LightCycler were converted into rRNA gene copies per sample area (gene copies/cm^2) for *Pseudoalteromonas*, and the relative fraction of *Pseudoalteromonas* rDNA per total *Eubacteria* rDNA was calculated as a percentage.

To determine the detection limit of our *Pseudoalteromonas*-specific RTQ-PCR assay, foreign DNA from salmon testes (ST) was introduced in the RTQ-PCR. ST DNA was added to the reaction mix to a final concentration of 20 ng/PCR tube. *P. tunicata* 16S rDNA template (standard) was added in the following quantities: 1.4, 14, 140, and 1,400 fg/PCR tube. The *Pseudoalteromonas*-specific RTQ-PCR was performed and analyzed as described above.

***Pseudoalteromonas*-specific PCR-DGGE.** A *Pseudoalteromonas*-specific PCR-DGGE protocol was developed based on the primers Eub341F-GC and Psalt815R (Table 1). The reaction mixture for PCR amplification contained 36.5 μl of dH_2O (Sigma), 5 μl of buffer (100 mM Tris-HCl, 750 mM KCl, 15 mM $MgCl_2$ [pH 8.8]), 5 μl of 10× (125 μM [each]) deoxynucleoside triphosphate (Invitrogen, Leek, The Netherlands), 1 μl of each primer (50 pmol/μl), 0.5 μl of *Taq* polymerase (5,000 U/ml; Pharmacia Biotech, Uppsala, Sweden), and 1 μl of template DNA (diluted 1:10 from raw extract). PCR amplification was carried out in a DNA thermocycler (PT-200 Peltier thermal cycler; MJ Research). The program consisted of an initial denaturation step for 60 s at 93°C followed by 40 cycles, with each cycle consisting of a 30-s denaturing step at 92°C, a 60-s annealing step at 62°C, and a 45-s (adding 1 s/cycle) extension step at 72°C. The reaction was completed by a 5-min final extension step at 72°C. To evaluate the PCR, products were loaded on a 2% Nusieve 3:1 agarose gel (FMC Bioproducts), and the results were evaluated and documented as described above.

Pseudoalteromonas amplified rDNAs were separated by DGGE according to their melting behavior. The D-GENE DGGE system from Bio-Rad was used with an 8% acrylamide gel and a denaturating gradient ranging from 25 to 65% (100% denaturant was 7 M urea and 40% [vol/vol] formamide). DGGE was optimized to run at a constant voltage of 200 V at 60°C for 7 h for maximum band separation. Bands of interest were excised from the gel and eluted in 50 μl of DNA buffer (10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA [pH 7.5]). Before sequencing, each band was subsequently reamplified and run again on a DGGE gel to confirm identical melting behavior and to verify that only one band was present. Primers used for reamplification in an attempt to sequence the DGGE bands lacked a GC clamp. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) prior to sequencing.

Cloning *Pseudoalteromonas* 16S rDNA. A cloning approach was used to further assess the diversity of *Pseudoalteromonas* species in samples found to have a high *Pseudoalteromonas* diversity in the DGGE analysis. DNA from seawater and a *Stylinos* sp. was amplified with the *Pseudoalteromonas*-specific primers (Eub341F-Psalt815R), both giving products of the correct length. These PCR products were purified with the QIAquick PCR purification kit (QIAGEN GmbH) and used for cloning. They were ligated into a pCR-XL-TOPO vector and transformed into ONE SHOT *Escherichia coli* cells following the manufacturer's directions (TOPO XL PCR cloning; Invitrogen). For each of the environmental samples, 10 clones were randomly picked and screened for an insert of the correct length by colony PCR amplification with the M13F(−20) and M13R primers provided with the kit. Plasmids were isolated and cleaned with a QIAprep spin miniprep kit (QIAGEN GmbH) prior to sequencing.

Sequencing environmental clones and DGGE bands. To identify the sequence composition of the DGGE bands and isolated plasmids, automated sequencing was performed. Purified PCR products from DGGE bands and plasmids with the correct-size inserts were sequenced directly with an ALFexpress DNA sequencer with a Thermosequenase fluorescence sequencing kit (Pharmacia). Previously described protocols for direct sequencing of PCR products and plasmids were followed (27, 45).

Phylogenetic analysis of partial sequences. Partial small-subunit rRNA sequences (Eub341F-Psalt815R) were obtained by *Pseudoalteromonas*-specific PCR-DGGE and cloning and analyzed by three different phylogenetic approaches. (i) An initial screen of the partial sequences was performed with the online sequence match function in the Ribosomal Database (RDP II; http://rdp.cme.msu.edu/html/) to roughly confirm the sequence identity. (ii) The partial sequences were thereafter imported to the latest ARB database (June 2002; http://www.arb-home.de). Partial sequences were aligned with the Fast Aligner (version 1.03) tool in the ARB Editor 4, and after manually inspecting the alignment, the sequences were added to the existing tree by using parsimony criteria without changing the overall tree topology. For this purpose, a filter was built with the sequence-associated information tool in the ARB software package. (iii) Finally, the sequences were aligned with the online sequence aligner (RDP II; http://rdp.cme.msu.edu/html/) and imported into the latest RDP II database (August 2002) containing almost full-length aligned prokaryotic sequences. Sequences were handled with the latest version of BioEdit (16). The phylogenetic analysis of the genus *Pseudoalteromonas* was performed with PAUP* (version 4.0; D. L. Swofford, Sinauer Associates, Sunderland, Mass.). Different phylogenetic algorithms, i.e., maximum parsimony, distance matrix, and maximum-likelihood analyses, were applied to the data using default PAUP settings.

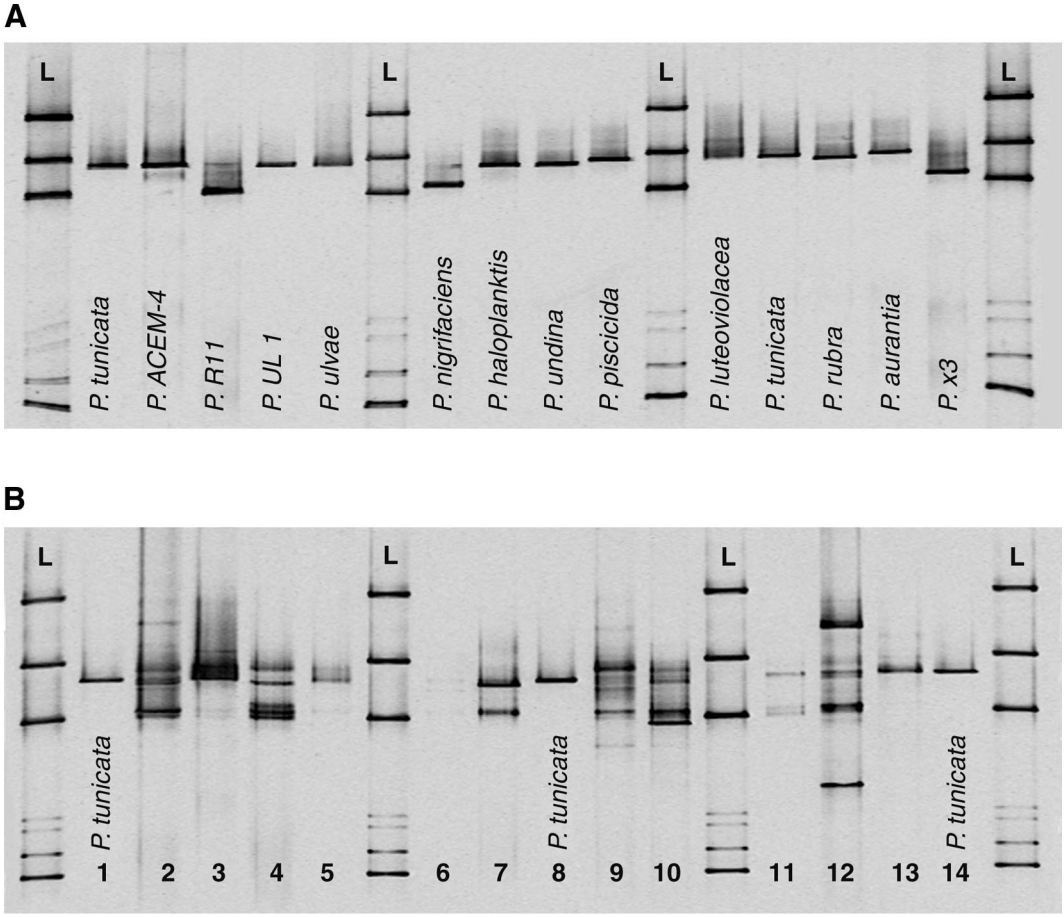


FIG. 1. *Pseudoalteromonas*-specific DGGE gels showing (A) the separation of 13 different *Pseudoalteromonas* pure cultures and (B) the *Pseudoalteromonas* diversity within 11 different marine samples: *P. tunicata* (lane 1), seawater (lane 2), rock (lane 3), *Codium fragile* (lane 4), *Dictyota dichotoma* (lane 5), *Ecklonia radiata* (lane 6), *Ulva lactuca* (lane 7), *P. tunicata* (lane 8), a *Corallina* sp. (lane 9), a *Stylinos* sp. (lane 10), an unidentified fouled ascidian (lane 11), *Cymbastela concentrica* (lane 12), *C. intestinalis* (lane 13), and *P. tunicata* (lane 14). Both gel pictures show the gradient interval from 40 to 55%. L, ladder.

RESULTS

Verification of primer specificity. The choice of primers for a PCR-based protocol will influence the results from an analysis of bacterial abundance or diversity in complex environmental samples (25, 33). Therefore, the specificity of the *Pseudoalteromonas*-specific primer set Eub341F-Psalt815R was tested extensively by using three different approaches: in silico, in vitro, and in situ testing.

Testing Psalt815R with the probe match function (RDP II; http://rdp.cme.msu.edu/html/), the probe match tool (ARB; http://www.arb-home.de), and BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) resulted in unique *Pseudoalteromonas* hits. A complete match with the primer Psalt815R was found in sequences from *P. aurantia*, *P. citrea*, *P. tunicata, P. ulvae*, and three identical green-pigmented strains isolated from *Ulva lactuca*, *Pseudoalteromonas* strains UL1, UL14, and UL15. If one mismatch (1 out of 22 nucleotides) was allowed in the primer search, all *Pseudoalteromonas* species were represented in the database search.

Twenty pure cultures were used to investigate whether this single-primer mismatch had any influence on the amplification of different *Pseudoalteromonas* species or if any false-positive strains were amplified in the *Pseudoalteromonas*-specific RTQ-PCR. A previously published *Pseudoalteromonas*-specific FISH probe, PSA184 (16S rRNA based), designed and tested by Eilers et al. (12), was reported to include the genus *Colwellia*. The genera *Colwellia*, *Alteromonas*, and *Shewanella* are all closely related to *Pseudoalteromonas* (14) and were therefore included in the analysis. The results demonstrated that all 13 *Pseudoalteromonas* species tested were positive and the 7 other species (*Agrobacterium*, *Alteromonas*, *Colwellia*, *Photobacterium*, *Pseudomonas*, *Shewanella*, and *Vibrio*) were negative by the *Pseudoalteromonas*-specific PCR after 25 cycles.

The 13 different *Pseudoalteromonas* species were amplified by the genus-specific PCR for DGGE (Fig. 1A). Very similar band positions were observed for the 13 *Pseudoalteromonas* sequences, and only small differences in mobility could be identified by using high-resolution pictures. A DGGE analysis of 11 different environmental samples showed bands with migration positions similar to those of the analyzed pure cultures of *Pseudoalteromonas* (Fig. 1B). From DGGE, a total of five

bands were sequenced. The sequenced bands from *C. intestinalis* (one band each from two individuals) and *Ulva lactuca* (one band from one individual and two bands from another individual) all belonged to the genus *Pseudoalteromonas* (Fig. 2).

A *Pseudoalteromonas*-specific cloning approach was used to obtain environmental *Pseudoalteromonas* sequences from seawater and *Stylinos* samples, which both represent samples with potentially higher *Pseudoalteromonas* diversity as shown on the DGGE (Fig. 1B). All nine sequences obtained from seawater clones and all eight sequences obtained from *Stylinos* clones could be placed within the *Pseudoalteromonas* tree (Fig. 2). Of the 22 analyzed environmental sequences obtained from our *Pseudoalteromonas*-specific PCR, all belonged to the genus *Pseudoalteromonas* when the ARB tree method (Fig. 2) was used. When the PAUP approach was used, two of the *Stylinos* clones fell in the closely related group *Oceanospirillum*.

Eukaryotic association of *Pseudoalteromonas* species. The maximum parsimony tree of the genus *Pseudoalteromonas* includes the newly available 16S rDNA sequences (Fig. 2). To investigate the distribution of the genus in the environment, a literature search was carried out with previously characterized *Pseudoalteromonas* species. This survey confirmed the notion that a high proportion of the described *Pseudoalteromonas* species have been isolated from eukaryotic hosts or other living surfaces in the marine environment (Fig. 2, underlined species in the tree). This physiological link to higher marine organisms was represented in all major branches of the tree.

Detection limits of RTQ-PCR and post-PCR analyses. The *Pseudoalteromonas*-specific RTQ-PCR assay had a linear range of detection spanning six orders of magnitude (Fig. 3). The detection limit of the *Pseudoalteromonas*-specific RTQ-PCR protocol in the presence of nontarget DNA was evaluated by adding ST DNA (20 ng/sample) to the master mix prior to amplification. *Pseudoalteromonas* 16S rDNA templates could be measured down to 1.4 fg of DNA/PCR tube (Fig. 4A). This gives a detection limit of 1,000 gene copies (1.4 fg) that could be detected in the presence of 20 ng of ST DNA. If no ST DNA was added to the master mix, as few as 10 gene copies (0.014 fg) of *Pseudoalteromonas* 16S rDNA were detected (data not shown). Running a PCR containing no *Pseudoalteromonas* 16S rDNA, with only ST DNA as template, gave a false-positive fluorescence signal similar in intensity to the signal obtained when 14 fg of *Pseudoalteromonas* 16S rDNA was added (Fig. 4A). The false-positive signal was investigated with a post-PCR analysis including inspection of an agarose gel and a melting curve analysis to identify products of non-*Pseudoalteromonas* origin. The agarose gel showed no specific product for the ST DNA sample in lane 5 (Fig. 4B), and no *Pseudoalteromonas* melting peak at 87.3°C could be identified for the false-positive signal (Fig. 4C). Such post-PCR analyses were performed on all quantified environmental samples in this study.

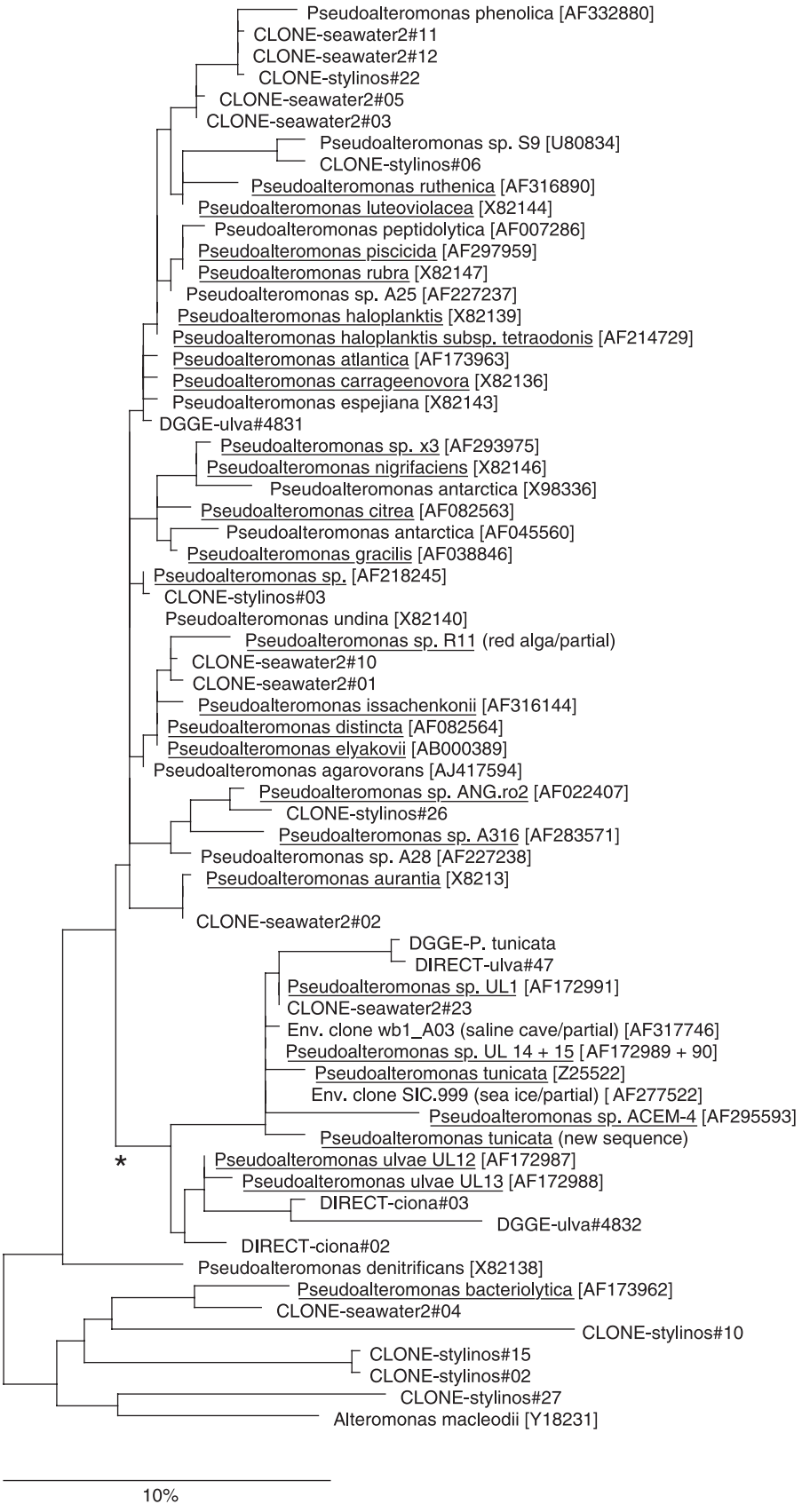
Robustness of the RTQ-PCR assay. To be able to compare results from the two different RTQ-PCR protocols (for *Pseudoalteromonas* and *Eubacteria*) and across different sample types, an analysis of the obtained standard curves was performed for each run (Table 2). The variation in the PCR amplification efficiency (E) for all 12 recorded runs was $<2\%$ relative standard deviation. The mean error, indicating tube-to-tube variation in the setup, was also found to be low for all LightCycler runs (between 0.08 and 0.17). A mean error value of 0.6 corresponds to a concentration deviation as high as 50%, according to the documentation that comes with the LightCycler. Regression coefficients for the 12 standard curves were all excellent: R was 1.00. These results show that both RTQ-PCR protocols were robust for the three different sample types. Results from different runs could therefore be compared and absolute and relative abundance of *Pseudoalteromonas* rDNA could be calculated.

Abundance of *Pseudoalteromonas* species in three marine samples. The genus-specific RTQ-PCR developed in the present study has the ability to determine both the absolute and the relative abundance of *Pseudoalteromonas* spp. 16S rRNA gene copies in complex environmental samples. Three sample types from marine waters around Aarhus, Denmark, were investigated for *Pseudoalteromonas* abundance: *Ulvaria fusca*, *Ulva lactuca*, and *C. intestinalis*. It was possible to calculate the relative abundance of *Pseudoalteromonas* rDNA in the total eubacterial rDNA since the two different RTQ-PCR protocols had identical amplification efficiencies (Table 2). Figure 5 shows the fractions of *Pseudoalteromonas* rDNA out of the total eubacterial rDNA abundance based on raw data from the LightCycler. *Ulva lactuca* contained the highest relative *Pseudoalteromonas* abundance (1.55%), followed by *C. intestinalis* (0.10%) and *Ulvaria fusca* (0.06%) (Table 3). The calculated average absolute values are based on (i) the measured gene copy number from the LightCycler, (ii) the total amount of extracted DNA from the sample, (iii) the amount of DNA used in the RTQ-PCR (5 ng), and (iv) the total area of the extracted sample. The absolute *Pseudoalteromonas* abundance values were 1.3×10^6 gene copies/cm^2 for *Ulva lactuca*, 2.5×10^5 gene copies/cm^2 for *C. intestinalis*, and 1.0×10^5 gene copies/cm^2 for *Ulvaria fusca*.

DISCUSSION

Members of the genus *Pseudoalteromonas* carry a number of remarkable physiological traits. Some *Pseudoalteromonas* species cause disease in fish, produce potent toxins, and excrete degrading agarolytic enzymes, while others appear less virulent and may even be beneficial to their natural hosts (23). To be able to study the community ecology and abundance of the genus *Pseudoalteromonas* in the marine environment, an RTQ-PCR protocol was developed, optimized, and applied in this study.

Primer evaluation. The *Pseudoalteromonas*-specific primer set was tested at three different levels (in silico, in vitro, and in situ). All 22 sequenced clones and DGGE bands belonged to the genus *Pseudoalteromonas* when analyzed with the ARB phylogenetic approach. When using the PAUP calculation method, two identical clones (of 22) obtained from a marine sponge *Stylinos* sp. belonged to the closely related group *Oceanospirillum*. This is not unexpected because sponge tissue in general contains a high total bacterial abundance and diversity (46, 47). Recent papers have successfully included the in situ primer evaluation approach in studies on *Bacillus* and *Burkholderia* diversity in different soils (13, 40) and in studies on the abundance and diversity of ammonia-oxidizing bacteria (4, 38).


Pseudoalteromonas phenolica [AF332880]
CLONE-seawater2#11
CLONE-seawater2#12
CLONE-stylinos#22
CLONE-seawater2#05
CLONE-seawater2#03
Pseudoalteromonas sp. S9 [U80834]
CLONE-stylinos#06
Pseudoalteromonas ruthenica [AF316890]
Pseudoalteromonas luteoviolacea [X82144]
Pseudoalteromonas peptidolytica [AF007286]
Pseudoalteromonas piscicida [AF297959]
Pseudoalteromonas rubra [X82147]
Pseudoalteromonas sp. A25 [AF227237]
Pseudoalteromonas haloplanktis [X82139]
Pseudoalteromonas haloplanktis subsp. tetraodonis [AF214729]
Pseudoalteromonas atlantica [AF173963]
Pseudoalteromonas carrageenovora [X82136]
Pseudoalteromonas espejiana [X82143]
DGGE-ulva#4831
Pseudoalteromonas sp. x3 [AF293975]
Pseudoalteromonas nigrifaciens [X82146]
Pseudoalteromonas antarctica [X98336]
Pseudoalteromonas citrea [AF082563]
Pseudoalteromonas antarctica [AF045560]
Pseudoalteromonas gracilis [AF038846]
Pseudoalteromonas sp. [AF218245]
CLONE-stylinos#03
Pseudoalteromonas undina [X82140]
Pseudoalteromonas sp. R11 (red alga/partial)
CLONE-seawater2#10
CLONE-seawater2#01
Pseudoalteromonas issachenkonii [AF316144]
Pseudoalteromonas distincta [AF082564]
Pseudoalteromonas elyakovii [AB000389]
Pseudoalteromonas agarovorans [AJ417594]
Pseudoalteromonas sp. ANG.ro2 [AF022407]
CLONE-stylinos#26
Pseudoalteromonas sp. A316 [AF283571]
Pseudoalteromonas sp. A28 [AF227238]
Pseudoalteromonas aurantia [X8213]
CLONE-seawater2#02
DGGE-P. tunicata
DIRECT-ulva#47
Pseudoalteromonas sp. UL1 [AF172991]
CLONE-seawater2#23
Env. clone wb1_A03 (saline cave/partial) [AF317746]
Pseudoalteromonas sp. UL 14 + 15 [AF172989 + 90]
Pseudoalteromonas tunicata [Z25522]
Env. clone SIC.999 (sea ice/partial) [AF277522]
Pseudoalteromonas sp. ACEM-4 [AF295593]
Pseudoalteromonas tunicata (new sequence)
*
Pseudoalteromonas ulvae UL12 [AF172987]
Pseudoalteromonas ulvae UL13 [AF172988]
DIRECT-ciona#03
DGGE-ulva#4832
DIRECT-ciona#02
Pseudoalteromonas denitrificans [X82138]
Pseudoalteromonas bacteriolytica [AF173962]
CLONE-seawater2#04
CLONE-stylinos#10
CLONE-stylinos#15
CLONE-stylinos#02
CLONE-stylinos#27
Alteromonas macleodii [Y18231]
10%

Post-PCR controls for RTQ-PCR. RTQ-PCR results have to be interpreted with robust post-PCR analyses to avoid false positives in the data set (Fig. 4). Such controls are critical to the results, especially if abundance values from different environmental matrices are compared with each other on a nonroutine basis, such as samples from the marine environment, activated sludge and soils (5, 18). In contrast, quantitative PCR-based studies that are focused on a few homogenous sample types, such as hospital or drinking water samples (7, 49) analyzed on a high-throughput system, might only require these stringent post-PCR controls in random checks.

Another issue that has to be considered is the nature of the SYBR Green quantification approach. All dsDNA are measured when using the SYBR Green approach for quantification in RTQ-PCR (17). This may introduce errors if unspecific PCR products and artifacts (e.g., primer-dimers) are measured together with the specific product of interest. Primer concentrations should always be optimized to give the lowest amount of primer-dimer. In the RTQ-PCR protocol, the fluorescent signal was acquired at 84°C after each cycle, which is above the primer-dimer melting point of 78°C and below the melting point value for the measured dsDNA of interest (Fig. 4C). This ensured that primer-dimers were not included in the measured population of double-stranded target DNA. Biases due to unspecific PCR products were avoided by inspecting melting curves and amplified PCR products on an agarose gel after each RTQ-PCR run.

Abundance assessment of *Pseudoalteromonas* species. The RTQ-PCR assay had a linear range of detection spanning six orders of magnitude (Fig. 3). While using DNA from one *Pseudoalteromonas* species (*P. tunicata*) in our standard curve, we assumed that the amplification efficiency for *P. tunicata* is equal to that of the mixed pool of 16S rRNA *Pseudoalteromonas* genes in the analyzed samples. This ought to be a reasonable assumption, given the low sequence variation (Fig. 1A) and short phylogenetic distance (Fig. 2) observed among the different *Pseudoalteromonas* species.

The RTQ-PCR detection limit was determined in the presence of nontarget DNA from ST (20 ng), with a detectable signal as low as 1.4 fg of DNA (1,000 gene copies). Detection limits of various RTQ-PCR protocols are often referred to as the smallest amount of DNA that can be detected against the standard curve with no nontarget DNA present (30, 39). Such detection limits are often in the range of a few gene copies. A more relevant measure is the environmental detection limit of the target DNA in the presence of nontarget DNA. This measure is relevant since target and nontarget DNA are always coextracted when studying a complex pool of genomic DNA from environmental samples. In samples with a low abundance of the target organism, the environmental detection limit would be of most interest if the background concentration of nontarget DNA were set at an environmentally realistic level.

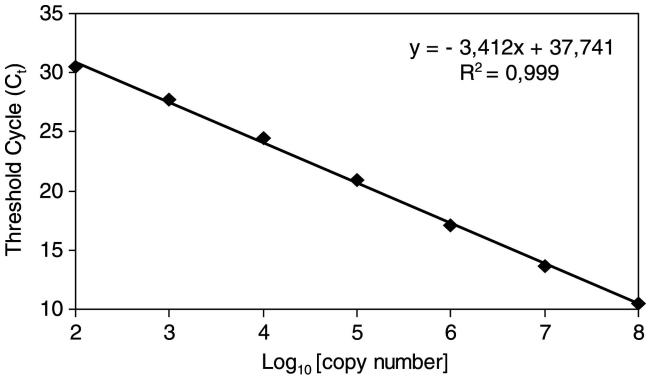


FIG. 3. Linear relationship between threshold cycles (C_t) and the input copy number of 16S rDNA from *P. tunicata*. The standard curve was obtained with the *Pseudoalteromonas*-specific RTQ-PCR protocol. Linear regression ($R^2 = 0.999$) results in an equation of $y = -3.4x + 37.7$.

The highest relative *Pseudoalteromonas* abundance was found on *Ulva lactuca* (1.55%). This abundance was 25 times higher than that of *Pseudoalteromonas* on the other green alga analyzed, *Ulvaria fusca* (0.06%), and 15 times higher than that for the tunicate *C. intestinalis* (0.10%). To our knowledge, no estimates of the abundance of *Pseudoalteromonas* on living marine surfaces have been published. However, Eilers et al. (12) performed a FISH study of the in situ abundance of pelagic bacteria in the North Sea and found that the abundance of *Pseudoalteromonas* spp. 16S rRNA (including the closely related genus *Colwellia*) relative to total eubacterial abundance was <1.0%. While this number is close to the detection limit of the FISH method, the results convey that *Pseudoalteromonas* species also exist in the open waters in measurable quantities. *Pseudoalteromonas* species that have been isolated from seawater samples include *P. denitrificans*, *P. espejiana*, *P. peptidolytica*, and *P. undina*.

As discussed by Lyons et al. (32), the abundance assessment could be determined either as the absolute number of gene copies in the sample or as the number of *Pseudoalteromonas* rRNA genes relative to the total eubacterial rRNA gene abundance. The use of relative abundance values has the advantage that adjustments are made for the variation obtained by using the different sampling protocols and for different sample types, which allows for comparisons between samples (1, 5, 42). The high variation in relative abundance between individual samples which was also found for *U. lactuca* would therefore reflect a true variation and not a variation due to artifacts from sampling or PCR. This variation may be due to such factors as

FIG. 2. Phylogenetic tree for genus *Pseudoalteromonas* based on almost complete 16S rRNA gene sequences. The maximum parsimony analysis was performed with standard settings in ARB. Phylogenetic affiliations of organisms represented by partial 16S rRNA gene sequences were reconstructed by applying parsimony criteria without changing the overall tree topology. DIRECT, sequences obtained via direct sequencing; DGGE, sequences obtained via DGGE bands cut out from the gel; CLONE, sequences obtained via cloning. DGGE-*P. tunicata* was a DGGE band cut from a pure culture of *P. tunicata* as a control. Accession numbers are in brackets. Underlined strains have been reported to be associated with eukaryotes. The asterisk indicates the subgroup containing the antifouling bacteria *P. tunicata* and *P. ulvae*.

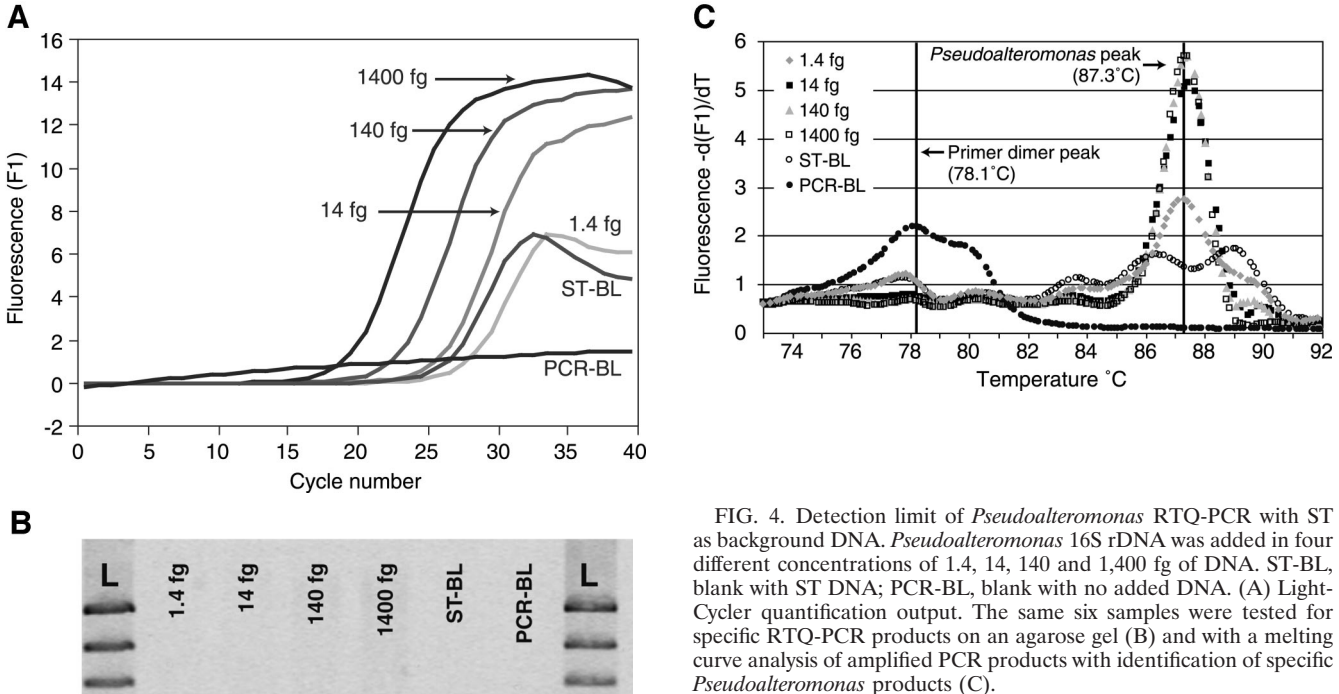


FIG. 4. Detection limit of *Pseudoalteromonas* RTQ-PCR with ST as background DNA. *Pseudoalteromonas* 16S rDNA was added in four different concentrations of 1.4, 14, 140 and 1,400 fg of DNA. ST-BL, blank with ST DNA; PCR-BL, blank with no added DNA. (A) LightCycler quantification output. The same six samples were tested for specific RTQ-PCR products on an agarose gel (B) and with a melting curve analysis of amplified PCR products with identification of specific *Pseudoalteromonas* products (C).

plant age and physical condition but was not further investigated in this study.

However, when the 16S rRNA gene is used for any quantification attempts on complex environmental samples, the issue of variation in the number of rRNA operons in different prokaryotes and natural environments and its impact on estimates of absolute and relative abundance also has to be addressed (28). This issue may introduce some variation in the abundance values obtained from different sample types containing *Pseudoalteromonas* populations with different levels of rRNA operon numbers. Another issue that derives from the unknown number of rRNA operons within the *Pseudoalteromonas* genome is the conversion from gene copies to actual cell numbers. As long as the number of operons is not known, the conversions to actual cell numbers have to be interpreted with caution (29).

Phylogeny and distribution of *Pseudoalteromonas* species. It was found that *Pseudoalteromonas* rRNA genes could be amplified from all the investigated marine samples used for primer evaluation by the genus-specific PCR-DGGE (Fig. 1B). This finding suggests a widespread distribution of the genus in the marine environment (e.g., on macroalgae, animals, and rocks and in seawater). This finding is in good agreement with published data describing habitats where the genus has been isolated. A literature study showed that a high proportion of the described *Pseudoalteromonas* species had been isolated from eukaryotic hosts. In the phylogenetic analysis, it was shown that these species were distributed across all major branches of the *Pseudoalteromonas* tree (Fig. 2). This finding suggests that associations with eukaryotic hosts reflect a physiological characteristic for the whole genus and not only a subset of species.

A distinct subgroup of the *Pseudoalteromonas* genus contains *P. tunicata* and *P. ulvae* (indicated in Fig. 2). These species have been isolated from the unfouled hosts *Ulva lactuca* and *C. intestinalis* (11, 24). In a study by Holmström et al. (21), these bacteria were found to display the highest level of antifouling activities out of the 10 different *Pseudoalteromonas* species tested. In this study, we found two sample types with DGGE bands that belonged to the *P. tunicata* and *P. ulvae* subgroup (Fig. 2). In total, four *Pseudoalteromonas* DGGE bands (ciona no. 2, ciona no. 3, ulva no. 47, and ulva no. 4832) were placed in this subgroup, originating from two individual *C. intestinalis* and two individual *Ulva lactuca* organisms. Hence, we have demonstrated that *Pseudoalteromonas* sequences obtained from these two unfouled hosts had the same identity as previously isolated bacteria from the same hosts. More extensive testing, including a large sampling regimen analyzed with molecular methods at the genus and subgroup levels, are required to arrive at any conclusions about the link

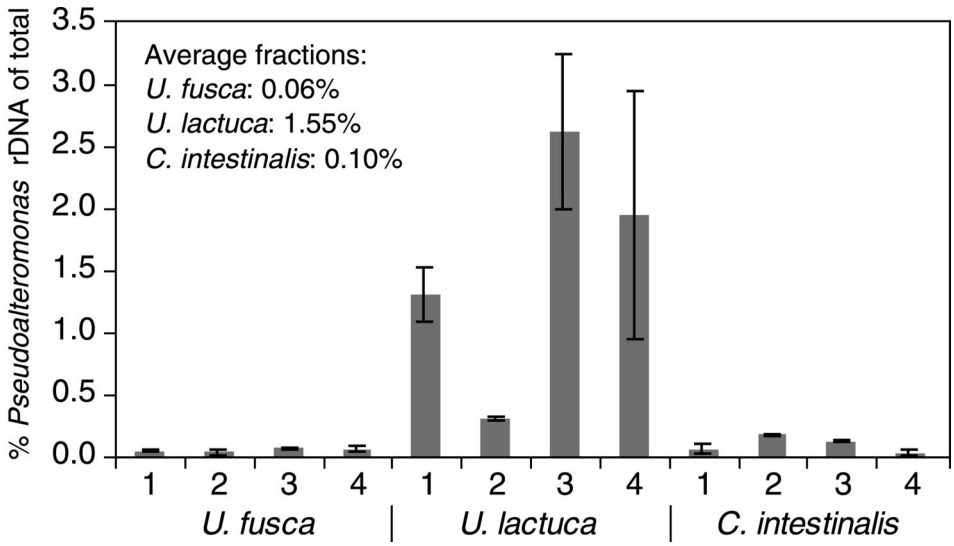


FIG. 5. Relative abundance of *Pseudoalteromonas* rDNA in total *Eubacteria* rDNA for four (1, 2, 3, and 4) individual higher marine organisms each of three different species. Error bars represent the standard deviations from duplicate RTQ-PCR runs.

between the physiologically distinct *P. tunicata* and *P. ulvae* subgroup and its antifouling properties.

Conclusions. Members of the genus *Pseudoalteromonas* were found in all the environmental samples analyzed in this study, suggesting a widespread distribution of the genus in the marine environment. With extensive primer testing at three different levels (in silico, in vitro, and in situ), we have shown that the *Pseudoalteromonas*-specific primers were unique for the genus and reliable for assessing abundance and diversity in different complex marine samples (e.g., seawater, rocks, macroalgae, and marine animals). With the optimized *Pseudoalteromonas*-specific RTQ-PCR, we enumerated both the absolute and relative abundance of *Pseudoalteromonas* in three different marine sample types. The relative abundance of *Pseudoalteromonas* species was found to be 1.55% for *Ulva lactuca*, 0.10% for *C. intestinalis*, and 0.06% for *Ulvaria fusca*. The RTQ-PCR method was robust over a range of six orders of magnitude and could detect target DNA levels as low as 1.4 fg of DNA in the presence of 20 ng of nontarget DNA. Reliable RTQ-PCR results were obtained from the three sample types that were analyzed, and no biases were observed during post-PCR analyses. We recommend that the controls applied in this study be considered if RTQ-PCR abundance values are obtained from and are compared across different complex environmental matrices on a nonroutine basis.

TABLE 3. Abundance of *Pseudoalteromonas* 16S rDNA

Sample	*Pseudoalteromonas* rDNA (gene copies/cm^2)[a]	% *Pseudoalteromonas* rDNA of total bacterial rDNA[b]
Ulvaria fusca	$1.0\ (0.8) \times 10^5$	0.06
Ulva lactuca	$1.3\ (0.6) \times 10^6$	1.55
C. intestinalis	$2.5\ (1.8) \times 10^5$	0.10

[a] Average gene copy numbers were obtained from four individuals and two RTQ-PCR runs ($n = 8$) for each sample type (standard deviations shown in parentheses).

[b] Averaged data from Fig. 5 for four individuals of each marine organism.

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