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Marine Bacteria from Danish Coastal Waters Show Antifouling Activity against the Marine Fouling Bacterium *Pseudoalteromonas* sp. Strain S91 and Zoospores of the Green Alga *Ulva australis* Independent of Bacteriocidal Activity▽†

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The aims of this study were to determine if marine bacteria from Danish coastal waters produce antifouling compounds and if antifouling bacteria could be ascribed to specific niches or seasons. We further assess if antibacterial effect is a good proxy for antifouling activity. We isolated 110 bacteria with anti-*Vibrio* activity from different sample types and locations during a 1-year sampling from Danish coastal waters. The strains were identified as *Pseudoalteromonas*, *Phaeobacter*, and *Vibrionaceae* based on phenotypic tests and partial 16S rRNA gene sequence similarity. The numbers of bioactive bacteria were significantly higher in warmer than in colder months. While some species were isolated at all sampling locations, others were niche specific. We repeatedly isolated *Phaeobacter gallaeciensis* at surfaces from one site and *Pseudoalteromonas tunicata* at two others. Twenty-two strains, representing the major taxonomic groups, different seasons, and isolation strategies, were tested for antiadhesive effect against the marine biofilm-forming bacterium *Pseudoalteromonas* sp. strain S91 and zoospores of the green alga *Ulva australis*. The antiadhesive effects were assessed by quantifying the number of strain S91 or *Ulva* spores attaching to a preformed biofilm of each of the 22 strains. The strongest antifouling activity was found in *Pseudoalteromonas* strains. Biofilms of *Pseudoalteromonas piscicida*, *Pseudoalteromonas tunicata*, and *Pseudoalteromonas ulvae* prevented *Pseudoalteromonas* S91 from attaching to steel surfaces. *P. piscicida* killed S91 bacteria in the suspension cultures, whereas *P. tunicata* and *P. ulvae* did not; however, they did prevent adhesion by nonbactericidal mechanism(s). Seven *Pseudoalteromonas* species, including *P. piscicida* and *P. tunicata*, reduced the number of settling *Ulva* zoospores to less than 10% of the number settling on control surfaces. The antifouling *alpP* gene was detected only in *P. tunicata* strains (with purple and yellow pigmentation), so other compounds/mechanisms must be present in the other *Pseudoalteromonas* strains with antifouling activity.

The bacterial colonization of marine surfaces and subsequent macrofouling is a ubiquitous phenomenon (20), and mature marine biofouling communities are complex and highly dynamic ecosystems which are difficult to eradicate once established (22). The biofouling of ship hulls (or other immersed structures) greatly increases the hydrodynamic drag and thereby leads to high fuel consumption (40) and CO_2 emissions. Costly mechanical processes coupled with toxic heavy metal-based paint containing, e.g., tin and copper, traditionally have been used to combat marine biofouling. Due to the nonspecific effects of heavy metal leaching, such paints are environmentally hazardous. An example is the very effective toxic compound tributyl tin, which is believed to contribute to the development of antimicrobial tolerance in marine organisms (35) and to cause imposex in some invertebrates (1, 16). Antifouling paints containing tin have been banned since 2003 and gradually removed from shipping fleets (27). Consequently, the search for antifouling compounds or principles that reduce or eliminate the attachment of marine organisms is intense (37). The key focus of the present study and other studies (7, 36) is to find environmentally friendly molecules or organisms (10) to replace the toxic/biocidal compounds being used to day. All macroorganisms in the marine environment have developed natural antifouling strategies, and our hypothesis is that any chemical components developed as part of a natural antifouling defense likely would be environmentally compatible.

Macroorganisms produce antifouling compounds, such as the halogenated furanones produced by the red algae *Delisea pulchra* (8), but they also rely on epiphytic bacteria as producers of antifouling compounds (11, 24, 36). From a biotechnological perspective, microorganisms are an exploitable source of antifouling compounds (37), hence we focused our search on marine bacteria. Some marine bacteria, primarily belonging to the *Pseudoalteromonas* genus, the *Vibrionaceae* family, and the *Roseobacter* clade, excrete compounds that can reduce bacterial biofilm formation and settlement by larger microorganisms on surfaces (6, 12, 38), suggesting their suitability as antifouling bacteria. However, a large-scale search for bacteria producing antifouling compounds is hampered by the fact that

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a high-throughput assay for screening large numbers of bacteria for antifouling capacity is not available. Typically, studies have focused on bacteria already known to inhibit the growth of other bacteria, such as the well-studied *Pseudoalteromonas tunicata* strain D2 (23, 25), or on a small subcollection of bacteria from a very specific niche (12, 15).

The purpose of the present study was to probe the antifouling potential of marine bacteria on a broader scale. We based our primary screen on the search for antibacterial activity, as this can be done in a high-throughput manner. Although we assume that bacteria producing antibacterial compounds also were likely to display antifouling capacity, it has never been determined that these characteristics are linked, i.e., if the search for antibacterial activity is a good proxy for antifouling activity. We deliberately took samples from different sources (water and surfaces), locations, and seasons to determine if bacteria with antibacterial and antifouling activity were associated with specific niches or seasons. We focused our sampling on bacteria isolated from live or inert marine surfaces, as other studies have indicated that marine bacteria associated with surfaces are more likely to display antibacterial activity than planktonic bacteria isolated from open waters (31, 33, 47).

MATERIALS AND METHODS

Sample collection. Seawater, swabs from marine surfaces (an area of between 1 and 10 cm^2 was swabbed), and samples such as seaweed, barnacles, and mussels were collected from 11 coastal sites in Denmark, representing different salinity and temperature conditions (Table 1). A total of 467 samples were analyzed and were derived from approximately two seawater and eight surface samples from each of the 11 samplings sites during different seasons (April, June, August, and November 2009 and February 2010). Water temperature and salinity were measured *in situ* with a handheld Professional Plus instrument (YS6050000; YSI, Yellow Springs, OH).

Total bacterial cell density in seawater. Total bacterial densities were determined using epifluorescence counts of SYBR gold-stained bacteria on 5-μm polycarbonate filters (no. K50BP02500; GE Water & Process Technologies) and 0.02-μm anodisc filters (no. 6809-6002; Whatman) as previously described (19). Cells were counted using an Olympus BX51 microscope with 460- to 490-nm excitation and >510-nm emission filters. Five fields were counted per sample.

Plate counts of culturable bacteria for all samples. Bacteria were extracted from solid samples of seaweed, algae, etc., and 10-fold serially diluted in 3% Instant Ocean (IO; Aquarium Systems Inc., Sarrebourg, France). Samples were divided into two groups according to their texture. Soft tissues, such as seaweeds, were homogenized using an Ultraturrax T25, whereas hard samples, such as small stones, barnacles, and mussel shells, were mixed by vortexing at maximum speed for 30 s. Seawater, swabs, and diluted samples also were 10-fold serially diluted in 3% IO and plated on 50% marine agar (MA; diluted with 1.5% IO; 212185; Difco). Plates were incubated at 20°C for 4 to 5 days, and colonies were counted to determine CFU (CFU/ml, CFU/g, or CFU/swab).

Enrichment of bacteria. From all water samples and from 10-fold-diluted surface/swab samples, 0.1 ml was mixed with 5 ml marine broth (279110; Difco) and incubated at 20°C for 2 weeks. In all of these enrichment samples, a biofilm formed on the glass at the air-water interface, and bacteria from these biofilm rings were streaked on MA and incubated at 20°C. Subsequently the bacteria were tested for antibacterial activity by replica plating against *V. anguillarum* strain 90-11-287 (41) as described below.

Selection of pigmented bacteria. Approximately 10 pigmented colonies were isolated from each sampling site at each sampling time after culturable plate count. Colonies were streaked on MA and tested for antibacterial activity against *V. anguillarum* strain 90-11-287 in a replica assay.

Antibacterial bioassay. Plate counts, colonies from enriched samples, and pigmented strains were tested for inhibitory activity against *V. anguillarum* strain 90-11-287 in agar-based assays in which *V. anguillarum* was incorporated into a 1.2% agar with 3% (wt/vol) IO salts, 0.3% (wt/vol) Bacto Casamino acids (product number 223050; BD, MD), and 0.4% (wt/vol) glucose as previously described (21). *V. anguillarum* strain 90-11-287 was selected as an indicator strain for antibacterial activity, as it is very sensitive to inhibitory compounds produced by other marine bacteria (19). Colonies causing a clearing zone in the turbid *V. anguillarum* layer were isolated from the original plate, restreaked, and stored at −80°C.

Identification of bacteria. Identification to the genus level was based on a combination of phenotypic characteristics (19) and 16S rRNA gene sequence analysis. Chromosomal DNA was purified from the marine bacteria grown for 3 days at 25°C in marine broth using the NucleoSpin tissue kit from Macherey-Nagel (M740952). The 16S rRNA genes were amplified using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACC TTGTTACGACTT) (19). Following gel electrophoresis, the PCR products were purified using the GFX PCR DNA and gel band purification kit from GE Healthcare (Buckinghamshire, United Kingdom). Sequencing was carried out by DNA Technology, Århus, Denmark, with 518F (CCAGCAGCCGCGGTAAT ACG) and 800R (TACCAGGGTATCTAATCC) as primers. Identification to the genus level was done using the BLAST-based program leBIBI (9) and NCBI databases. The inhibitory activities of all strains were retested in agar diffusion assays against *V. anguillarum* strain 90-11-287 after being frozen at −80°C for at least 3 months.

Presence of a known antifouling gene. All *Pseudoalteromonas* strains were tested for the presence of the *alpP* gene by PCR with primers previously described (42). Aliquots (2 μl) of purified DNA were applied to the following to give a 25-μl PCR mixture: 13 μl Brilliant II quantitative PCR master mix (2×) (Agilent Technology), 1 μl (12.5 μM) of primer alpP-1F and primer alpP-2R, and 8 μl sterile MilliQ water. The PCR amplification (15 min at 95°C and then 40 cycles of three steps consisting of 30 s at 92°C, 60 s at 55°C, and 30 s at 72°C, with a final extension of 5 min at 72°C) was performed with a 9800 Fast Thermal Cycler (Applied Biosystems). The size of the final PCR product was determined by electrophoresis in a 1% agarose gel.

Collection and sporulation of *Ulva australis*. *U. australis* cells were transported (for approximately 20 min) on ice after collection and then frozen at −20°C for half an hour. The plants were thawed and rinsed gently in sterile filtered seawater, placed in a beaker with sterile filtered seawater, and positioned close to a light source (desk lamp) to induce sporulation (13). The phototactic response of the zoospores was used to remove other unicellular organisms associated with the algal spores and to select spores with higher chances of survival (13). Spores were added to one side of a glass tray (20 cm long) containing sterile filtered seawater, and spores were allowed to migrate toward a light source at the other end. After 10 to 15 min, spores that reached the other side of the tray were collected.

Streptomycin resistance. Twenty-two isolates representing *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, and *Phaeobacter* were tested for antiadhesive properties. Apart from representing the major taxonomic groups, the isolates also represented different seasons and isolation strategies (plate count, enrichment, and pigmentation). The strains were tested for their ability to prevent the attachment of a marine biofilm-forming bacterium, *Pseudoalteromonas* strain S91 (45). Strain S91 is streptomycin resistant, and the 22 strains were tested for streptomycin resistance to determine if streptomycin-containing agar medium would allow us to differentiate between S91 and the potential antifouling bacteria. The strains were plated on marine agar containing 25, 50, 100, and 200 μg/ml of streptomycin and incubated at 25°C for up to 1 week.

Antiadhesive activity and growth inhibition. Adhesion to and prevention of adhesion to inert surfaces was tested using stainless steel coupons (5 by 5 by 1 mm). The reporter strain S91 was chosen as a marker for antiadhesive effect against marine bacteria, as it is a nonantagonistic marine environmental strain which attaches to both abiotic and marine biotic surfaces and produces extended biofilms (44). The strain also is streptomycin resistant, which allowed us to distinguish between S91 and the potential antifouling marine strains. We quantified bacteria on the steel coupons with removal by sonication, followed by plate count on marine agar with and without streptomycin. We have previously demonstrated in pure cultures that the bacterial numbers determined by sonication removal and subsequent plating agrees well with numbers determined by the direct staining of attached bacteria (3). *In situ* quantification by staining with different probes followed by fluorescence microscopy also could have been used but would require strain-specific probes and that the number of bacteria on the surface was quantifiable by microscopy.

Prior to use, the coupons were cleaned by soaking overnight in a 15% Deconex solution, rinsed, degreased with acetone, and sterilized by autoclaving. The coupons were tilted and placed individually in wells of a microtiter plate (no. 167008; Nunc). The 22 marine bacteria were cultured in marine broth for 3 days at 25°C and diluted by a factor of 1,000 in marine broth, and 200 μl was transferred to each well containing the coupons. The bacteria were allowed to grow and attach to the stainless steel surface for 3 days at 25°C before the coupons were washed twice in 2 ml of 3% IO, transferred to a new microtiter plate, and exposed to a marine fouling bacterium. The amount of biofilms

TABLE 1. Chemical and microbiological parameters in seawater collected during a 1-year survey from 11 coastal sites in Denmark

Sample site and collection time	Chemical parameter		Microbiological parameter[a]	
	Temp (°C)	Salinity (%)	Total cell count (log cells/ml)	Culturable counts (log CFU/ml)
Jyllinge Habour				
April 2009	12.2 ± 0.1	1.4 ± 0.0	6.60 ± 0.03	2.98 ± 0.02
June 2009	16.0 ± 0.0	1.4 ± 0.0	6.32 ± 0.06	3.74 ± 0.06
August 2009	18.8 ± 0.0	1.4 ± 0.0	6.74 ± 0.05	2.90 ± 0.00
November 2009	7.4 ± 0.1	1.5 ± 0.0	6.43 ± 0.07	2.87 ± 0.08
February 2010	ND[b]	ND	5.85 ± 0.02	2.65 ± 0.10
Gilleje				
April 2009	12.0 ± 0.4	1.7 ± 0.0	6.26 ± 0.03	3.38 ± 0.24
June 2009	14.2 ± 0.0	1.5 ± 0.1	6.54 ± 0.02	4.72 ± 0.05
August 2009	21.3 ± 0.3	1.7 ± 0.3	6.57 ± 0.03	3.00 ± 0.00
November 2009	8.6 ± 0.0	1.8 ± 0.1	5.47 ± 0.17	4.35 ± 0.04
February 2010	−1.0 ± 0.1	ND	5.87 ± 0.07	2.04 ± 0.06
Bellevue				
April 2009	13.7 ± 0.1	0.8 ± 0.1	6.41 ± 0.11	3.48 ± 0.06
June 2009	13.7 ± 0.0	1.0 ± 0.0	6.60 ± 0.06	3.65 ± 0.23
August 2009	18.8 ± 0.1	1.0 ± 0.0	6.69 ± 0.03	4.02 ± 0.08
November 2009	6.1 ± 0.0	1.0 ± 0.0	6.55 ± 0.04	3.98 ± 0.02
February 2010	−0.2 ± 0.1	1.0 ± 0.0	6.21 ± 0.02	2.81 ± 0.05
Limfjorden				
April 2009	9.5 ± 0.1	2.2 ± 0.3	6.87 ± 0.06	4.58 ± 0.23
June 2009	16.7 ± 0.1	2.3 ± 0.2	6.55 ± 0.06	4.45 ± 0.03
August 2009	17.4 ± 0.1	2.6 ± 0.0	6.80 ± 0.04	3.93 ± 0.00
November 2009	7.5 ± 0.0	2.0 ± 0.0	6.48 ± 0.03	5.22 ± 0.18
February 2010	ND	ND	4.96 ± 0.26*	3.72 ± 0.07*
North Sea (north Agger)				
April 2009	8.2 ± 0.0	3.3 ± 0.0	7.00 ± 0.04	4.77 ± 0.22
June 2009	12.3 ± 0.0	3.5 ± 0.0	6.24 ± 0.11	3.32 ± 0.13
August 2009	17.7 ± 0.0	2.9 ± 0.1	6.04 ± 0.06	3.77 ± 0.23
November 2009	9.5 ± 0.0	3.0 ± 0.3	5.67 ± 0.07	3.01 ± 0.00
February 2010	−0.9 ± 0.0	3.4 ± 0.0	5.76 ± 0.09	2.74 ± 0.04
North Sea (halfway Hvide sande)				
April 2009	11.2 ± 0.0	2.2 ± 0.0	6.92 ± 0.06	4.89 ± 0.02
June 2009	13.2 ± 0.0	2.1 ± 0.1	6.55 ± 0.03	4.67 ± 0.42
August 2009	16.7 ± 0.1	2.0 ± 0.1	6.68 ± 0.06	4.11 ± 0.13
November 2009	8.2 ± 0.0	2.0 ± 0.1	6.26 ± 0.05	3.06 ± 0.02
February 2010	0.0	2.4 ± 0.0	5.59 ± 0.04	2.72 ± 0.14
North Sea (south Blåvand)				
April 2009	14.9 ± 0.1	2.7 ± 0.3	6.85 ± 0.07	4.90 ± 0.02
June 2009	14.6 ± 0.1	2.7 ± 0.1	6.47 ± 0.05	4.09 ± 0.04
August 2009	17.8 ± 0.1	3.2 ± 0.0	6.40 ± 0.03	3.59 ± 0.24
November 2009	8.3 ± 0.0	3.1 ± 0.0	6.26 ± 0.06	3.08 ± 0.07
February 2010	−0.3 ± 0.0	3.0 ± 0.0	6.07 ± 0.04	3.38 ± 0.04
Middelfart				
April 2009	11.9 ± 0.4	2.1 ± 0.1	6.82 ± 0.05	4.62 ± 0.08
June 2009	15.3 ± 0.1	2.1 ± 0.0	6.70 ± 0.08	4.54 ± 0.06
August 2009	17.9 ± 0.1	2.3 ± 0.0	6.82 ± 0.05	4.21 ± 0.07
November 2009	8.9 ± 0.1	1.8 ± 0.0	6.53 ± 0.10	5.70 ± 0.31
February 2010	0.9 ± 0.0	1.1 ± 0.3	6.19 ± 0.02	3.87 ± 0.02
Korsør				
April 2009	13.7 ± 1.2	1.5 ± 0.0	6.19 ± 0.05	4.83 ± 0.10
June 2009	15.3 ± 0.1	1.6 ± 0.0	6.32 ± 0.11	3.65 ± 0.21
August 2009	18.9 ± 0.0	1.4 ± 0.0	6.62 ± 0.03	4.61 ± 0.20
November 2009	7.1 ± 0.1	1.2 ± 0.0	6.65 ± 0.07	3.04 ± 0.16
February 2010	0.6 ± 0.0	1.2 ± 0.0	6.24 ± 0.05	2.86 ± 0.26
Kalvehave Møn				
April 2009	15.3 ± 0.8	0.9 ± 0.0	6.63 ± 0.09	2.98 ± 0.34
June 2009	12.7 ± 0.0	0.9 ± 0.0	5.64 ± 0.21	5.29 ± 1.01
August 2009	20.4 ± 0.0	1.0 ± 0.0	6.72 ± 0.05	4.35 ± 0.21
November 2009	6.3 ± 0.4	1.0 ± 0.0	6.65 ± 0.03	4.07 ± 0.40
February 2010	ND	ND	ND	5.01 ± 0.00*
Stensballe Horsens				
April 2009	15.1 ± 0.4	2.1 ± 0.0	6.48 ± 0.15	5.42 ± 0.09
June 2009	19.5 ± 0.3	2.4 ± 0.1	6.19 ± 0.07	4.49 ± 0.22
August 2009	17.7 ± 0.0	2.3 ± 0.1	6.62 ± 0.06	5.26 ± 0.13
November 2009	7.7 ± 0.1	1.9 ± 0.0	5.10 ± 0.26	4.07 ± 0.40
February 2010	ND	ND	ND	5.26 ± 0.00*

[a] A 0.02-μm filter was used. *, measured on frozen water.
[b] ND, not determined.

formed on the stainless steel coupons prior to exposure to S91 was determined by sonication and plate counts as described below. The spontaneous streptomycin-resistant mutant S91 of the marine fouling bacteria *Pseudoalteromonas* sp. strain S9 (45) was grown for 3 days in marine broth and diluted by a factor of 10,000 in 3% sea salt (S9883; Sigma-Aldrich). Two hundred μl was transferred to each well in the microtiter plate containing the biofilm-coated coupons, and the bacterial adhesion of strain S91 was allowed to take place on both sites of the coupons. Samples of the suspension and of the steel plates were taken after 1, 4, and 24 h. The stainless steel coupons (with attached bacteria) were immersed in polystyrene tubes (Sterikin LDT; Bibby Sterin LDT, Stones, United Kingdom) containing 2 ml sterile 3% sea salt (Sigma). Bacteria were removed from the surface by sonication (4) and vortexed at maximum speed for 15 s to further facilitate removal. The samples were serially diluted in sterile 3% sea salt (Sigma), and colony counts of the total number of adhered bacteria and the number of adhered S91 were determined by plating on 50% marine agar and 50% marine agar containing 400 μg/ml streptomycin, respectively. The efficiency of the detachment procedure was verified by the SYBR gold staining of the steel coupons followed by fluorescence microscopy (3). Bacterial counts in the suspensions also were done. All adhesion assays were carried out in triplicate. *Phaeobacter* strain 27-4 (21) and *Pseudoalteromonas tunicata* D2 (23) were included as positive (antifouling) controls, and marine broth was used as a negative control.

***Ulva* zoospore adhesion and germination assay.** The effect of bacteria on algal zoospore settlement and germination was studied using the marine alga *U. australis*. It was collected prior to sporulation from rock surfaces located at Shark Point, Clovelly, Sydney, Australia. Algal spores were exposed directly to monospecies bacterial biofilms of the 22 strains. The marine bacteria were cultured in marine broth for 3 days at 25°C and diluted by a factor of 100, and 1 ml was transferred to a 24-multiwell culture plate (Sigma). The bacteria were allowed to grow and form biofilms on the plastic surfaces for 3 days at 25°C before the growth medium was discarded. The wells were washed twice with sterile filtered (0.22-μm pore size; Millex; Millipore, Carrigtwohill, Ireland) seawater prior to adding the spores. The amount of bacterial biofilms was assessed by the crystal violet staining of biofilms grown under conditions identical to those used for the spore settlement assay (29). One ml of the *Ulva* spore suspension (described above) was added to each well in the 24 multiwells coated with bacteria, and the plates were placed in darkness for 2 h to allow the even settlement of spores. The plates then were incubated at room temperature under natural light for 24 h (for spore settlement), 1 ml of fresh sterile seawater was added, and the incubation was extended for six more days (for germination).

The quantification of the number of settled spores and germination was done by counting 10 fields of vision under 40× magnification using an inverted light microscope (Zeiss). Treatments were compared to controls consisting of noncoated and marine broth-coated wells.

Statistical analysis. The comparison of the prevalence of inhibitory colonies isolated in the different sampling months (April, June, August, November, and February) was done using Pearson's chi-squared test. The comparison of antifouling activity against strain S91 was done by a *t* test comparison of log-transformed cell densities (CFU/ml or CFU/cm^2).

RESULTS

Sample collection. A total of 467 samples were analyzed from the 11 coastal sites in April, June, August, and November 2009 and February 2010. The average temperatures varied during the year, being just below 0°C in February and above 18°C in August (Table 1). Variation was seen between the sampling sites, and as expected the water temperatures in the fjords compared to coastal waters were higher in the summer months and lower in the winter months (Table 1). We were only able to measure temperature and salinity at 6 of 11 sampling sites in February due to snow and sea ice. The average salinity level from April to November for each sampling site varied from below 10 practical salinity units (psu) at the southeast cost of Denmark to above 30 psu at the northwest coast (Table 1). The variation between salinity measurements at the same sampling site over time was low (Table 1).

Total bacterial cell density and culturable bacterial counts. The total cell counts of the seawater were between 5.1 and 7.0 log cells per ml, of which approximately 0.4% were culturable on 50% marine agar. Large variations were observed in the culturable counts between the different locations at each sampling month (Table 1).

Seawater samples (105 in total) contained 3.9 ± 0.3 log CFU/ml of culturable bacteria, whereas numbers of culturable organisms were higher in swab samples (165 in total) and whole-surface samples (197 in total), 6.8 ± 1.3 and 6.9 ± 1.2 log CFU/g, respectively. Approximately 124,000 bacterial colonies, of which 19,000 were from water samples, 43,000 from swab samples, and 61,000 from whole surface samples, were replica plated and tested for inhibitory activity against *V. anguillarum* strain 90-11-287. Of these 300 colonies (1.6%) from seawater, 515 (1.2%) from swab samples and 505 (0.8%) from whole-surface samples were inhibitory against *V. anguillarum* strain 90-11-287 in the primary replica plating. Forty-one of the 1,320 inhibitory colonies were restreaked and identified as described below. These were chosen to represent the different isolation times, places, and sample types (surface and water samples).

TABLE 2. Influence of isolation strategy on marine bacteria from Danish coastal waters capable of inhibiting *Vibrio anguillarum* strain 90-11-287

Identification based on 16S rRNA gene sequence	No. of isolated *Vibrio anguillarum* inhibitory strains based on isolation method			
	Colony count	Pigmented colonies	Enrichment in MB	Total
Pseudoalteromonas	18	17	19	54
Vibrio	4	2	18	24
Phaeobacter	10	0	0	10
Photobacterium	0	0	8	8
Shewanella	0	1	1	2
Marinomonas	5	0	0	5
Other	4	1	2	7
Total	41	21	48	110

Enrichment of bacteria. Enrichment in marine broth for 2 weeks resulted in the development of a biofilm at the air-liquid interface. Streaking from 229 biofilms on 50% marine agar and replica plating resulted in 48 strains inhibiting *V. anguillarum* (Table 2). The inhibitory strains isolated after enrichment all were nonpigmented or had only a slight pigmentation (light yellow or orange). All of these 48 strains were included in further analyses.

Selection of pigmented bacteria. Since several pigmented marine bacteria display either antibacterial or antifouling activity (6), we also randomly isolated highly pigmented colonies from the 50% MA plates. A total of 294 strains were isolated, and 21 (7.1%) were inhibitory against *V. anguillarum*, and they all were isolated from surface samples. Seventeen of the 21 inhibitory strains were identified as *Pseudoalteromonas* and were white, yellow, orange, purple, brown, or black.

Identification of marine bacteria with antibacterial activity. One hundred ten strains inhibiting *V. anguillarum* were selected based on the size of inhibition zones in the replica plates against *V. anguillarum*, different colony morphology, and different pigmentation (see Table S1 in the supplemental material). Also, we ensured that strains were from different sample

types, times of sampling, and sampling places. The 110 strains consisted of 41 strains isolated from plate counts, 48 after selective enrichment and 21 selected based on pigmentation (Table 2). All antagonistic isolates were Gram-negative rods with positive oxidase and catalase reactions. Forty-five strains were intensely pigmented, being yellow, purple, orange, or black (21 of these were directly isolated due to their pigmentation). The similarity of 16S rRNA gene sequences identified the majority as *Pseudoalteromonas* (54 strains), *Vibrio* (24 strains), *Photobacterium* (8 strains), and *Phaeobacter* (10 strains). Eighty-seven strains retained inhibition upon retesting against *V. anguillarum*. It was mainly the nonpigmented *Pseudoalteromonas* strains and *Vibrio* strains isolated in November and February that lost their activity (data not shown).

Species identification of *Pseudoalteromonas* strains. Our subsequent findings (see below) indicated that some of the isolated *Pseudoalteromonas* strains had pronounced antifouling activity, and as stated in the introduction, this has been described previously for *P. tunicata*. The initial BLAST searches querying the 16S rRNA gene sequences of our *Pseudoalteromonas* strains were ambiguous (i.e., gave large numbers of hits with identical scores). A BLAST (http://blast.ncbi.nlm.nih.gov) search of our isolated *Pseudoalteromonas* strains against a compilation of *Pseudoalteromonas* type strain sequences retrieved from GenBank (the list of type strains was obtained from http://www.bacterio.cict.fr) therefore was done to identify isolates to the species level. The best type strain BLAST match identified 15 strains as *P. piscicida*, 5 strains as *P. tunicata* (being either dark purple or yellow on marine agar), and 2 strains as *P. ulvae* (Fig. 1). To group the bacteria according to 16S rRNA gene sequence similarity, a phylogenetic tree based on 52 of our isolated *Pseudoalteromonas* strains (for the last two of our strains the 16S rRNA samples were short, therefore these strains were left out of the analysis) and *P. tunicate* D2 (23) was done using *Salinispora arenicola* strain CNS-205 as an outgroup as previously described (47). The clustering of the phylogenetic analysis reflected both species and pigmentation (Fig. 1). The presence of pigmentation was indicative of a stable antibacterial activity against *V. anguillarum* strains except strain F51a-1 (identified as *P. aliena*), which was nonpigmented and had strong inhibitory activity (Fig. 1).

The *alpP* gene was detected in all strains that were identified by 16S rRNA gene similarity as being *P. tunicata* independently of their pigmentation (blackish or yellow).

Effect of spatial and seasonal variation on isolation of bioactive bacterial strains. The prevalence of inhibitory culturable bacteria varied by season (temperature), with the lowest prevalence being seen in the colder months. Of the approximately 124,000 bacterial colonies tested for inhibitory activity against *V. anguillarum*, 35,000 were isolated in April, 27,000 in June, 19,000 in August, 24,000 in November, and 18,000 in February. The inhibitory colonies constituted 1.5 (April), 1.2 (June), 1.6 (August), 0.5 (November), and 0.03% (February) of the populations. This monthly difference between the prevalence of inhibitory bacteria was highly significant in the Pearson's chi-squared test ($P < 0.001$). Antibacterial *Pseudoalteromonas* and *Vibrio* strains were isolated year-round, whereas antibacterial *Phaeobacter* and *Photobacterium* were detected only in August and November (Table 3). The *Pseudoalteromonas* species isolated varied according to season/water temper-

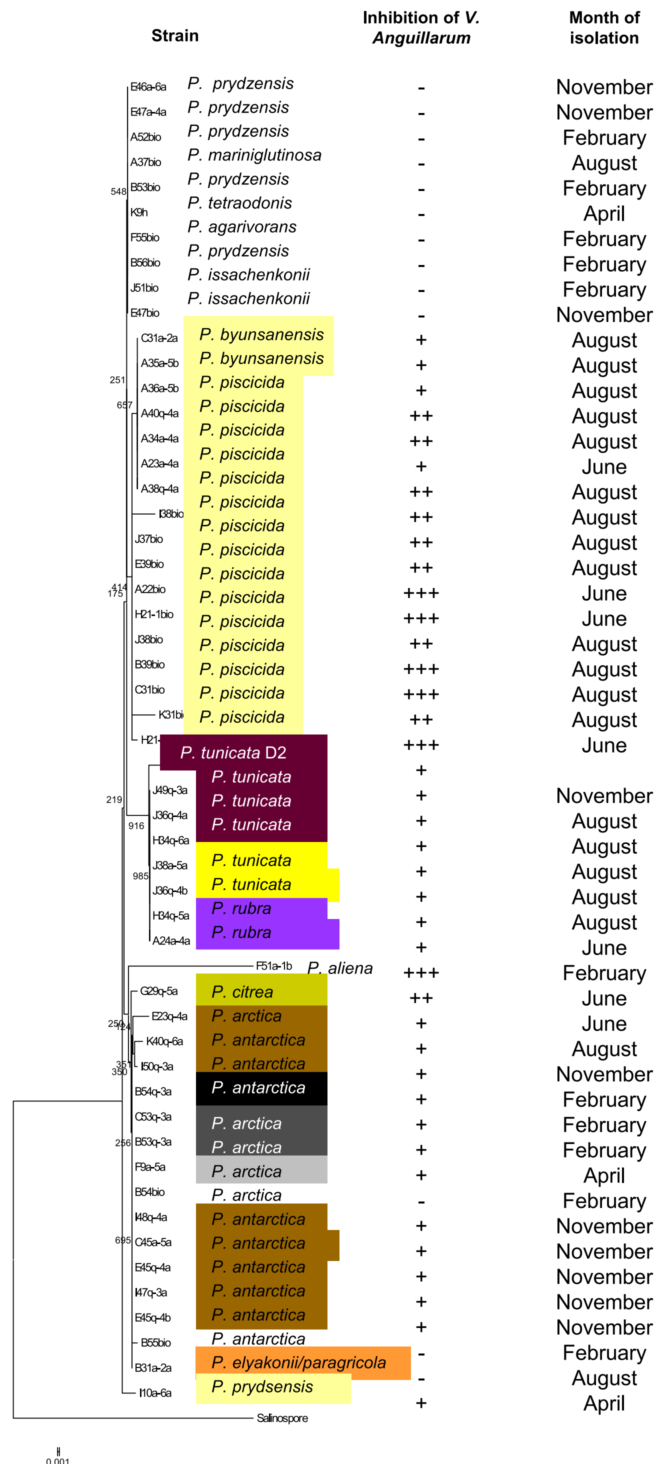

FIG. 1. Phylogenetic tree based on 16S-rRNA gene sequences of *Pseudoalteromonas* collected in Danish coastal waters. Sequences were aligned by MAFFT (default options), and the resulting alignment was used to generate a neighbor-joining tree in the MEGA4 software package (default settings; 1,000 bootstrap replicates). *Salinispora arenicola* CNS-205 (GenBank accession number CP000850; GeneID 5705939) was used as the outgroup. The suffix bio indicates that bacteria were isolated after enrichment in marine broth for 2 weeks. Zone diameters in well diffusion assays with *V. anguillarum* strain 90-11-287 were designated with the following symbols: ≤6 mm, −; 6.1 to 10 mm, +; 10.1 to 15 mm, ++; and >15 mm, +++. The well itself has a diameter of 6 mm. No inhibition of supernatants occurred.

TABLE 3. Influence of season on marine bacteria from Danish coastal waters capable of inhibiting *Vibrio anguillarum* strain 90-11-287

Identification based on 16S rRNA gene sequence	No. of *Vibrio anguillarum* inhibitory strains by time point[a]					
	April (12.5 ± 2.3)	June (14.8 ± 2.0)	August (18.5 ± 1.3)	November (7.8 ± 1.0)	February (−0.1 ± 0.7)	Total
Pseudoalteromonas	3	8	21	11	11	54
Vibrio	1	5	12	5	1	24
Phaeobacter	0	0	6	4	0	10
Photobacterium	0	1	5	2	0	8
Shewanella	0	1	0	0	1	2
Marinomonas	0	0	0	5	0	5
Other	1	1	2	2	3	7
Total	5	16	46	30	16	110

[a] Numbers in parentheses indicate temperatures (average ± standard deviation).

ature (Fig. 1). *P. piscicida* was frequently isolated in June and August, whereas *P. tunicata* was isolated in August and November (Fig. 1). *Pseudoalteromonas antarctica* and *P. arctica* were isolated mainly in November and February (Fig. 1).

There were geographical patterns in the isolation of antibacterial bacteria strains, since some genera were detected only in certain areas (Table 4). All 10 *Phaeobacter* strains were isolated from samples from Jyllinge harbor, whereas all except one of the *P. tunicata* strains were isolated from Møn. Both sites are characterized by waters with low turbulence and NaCl concentrations of 1.4 and 1.0%, respectively (Table 4).

Influence of isolation method on phylogenetic distribution of bioactive strains. Bacterial strains producing bioactive compounds were isolated with three different isolation strategies, (i) plate count, (ii) selection for pigmented strains, and (iii) enrichment for 2 weeks in marine broth, all followed by replica plating against *V. anguillarum*. These different isolation strategies resulted in different bacterial genera (Table 2). Enrichment in marine broth favored the growth of bacteria belonging to the *Vibrionaceae*, since 18 of 24 inhibitory *Vibrio* strains and all eight *Photobacterium* strains were isolated after enrichment (Table 2). The opposite was seen for *Phaeobacter* and *Marinomonas*, where all strains were found after direct plate counting (Table 2). Bacteria belonging to the *Pseudoalteromonas* group were isolated by all procedures; however, the vast majority of nonpigmented strains in this genus were isolated after enrichment (Fig. 1). Ten of 15 *P. piscicida* strains were isolated after enrichment (Fig. 1), indicating a strong ability of this species to outcompete other marine bacteria under these culturing conditions.

TABLE 4. Influence of site of isolation on marine bacteria from Danish coastal waters capable of inhibiting *Vibrio anguillarum* strain 90-11-287

Identification based on 16S rRNA gene sequence	No. of *Vibrio anguillarum* inhibitory strains from location[a]:											
	A	B	C	D	E	F	G	H	I	J	K	Total
Pseudoalteromonas	11	8	4	0	7	3	1	4	5	7	4	54
Vibrio	2	2	3	1	4	4	4	1	2	1	0	24
Phaeobacter	10	0	0	0	0	0	0	0	0	0	0	10
Photobacterium	1	1	0	0	0	0	0	2	1	2	1	8
Shewanella	0	1	0	0	0	1	0	0	0	0	0	2
Marinomonas	1	0	2	0	0	0	0	1	0	1	0	5
Other	0	0	0	0	1	1	0	1	0	2	2	7
Total	25	12	9	1	12	9	5	9	8	13	7	110

[a] Locations: A, Jyllinge harbor; B, Gilleje; C, Bellevue; D, Thisted; E, Agger; F, Hvide Sande; G, Blåvand; H, Middelfart; I, Korsør; J, Kalvehave at Møn; and K, Horsens.

Antiadhesive activity of antibacterial marine bacteria. Twenty-two strains were chosen (based on the criteria described in Materials and Methods) for antiadhesive studies against the marine fouling bacteria *Pseudoalteromonas* S91 and against the settlement and germination of *Ulva australis* zoospores. From the *Pseudoalteromonas* group we included different species and chose three *P. piscicida* strains, since this species repeatedly caused large and clear inhibition zones when replica plated against *V. anguillarum*, and it represented different degrees of pigmentation (yellow and very light yellow). We also included three strains of *P. tunicata* (two yellow and a dark purple strain), since pigmentation and antifouling properties have previously been linked in *P. tunicata* strain D2 (14). Twenty-one of the 22 marine strains with inhibitory activity against *V. anguillarum* attached to and formed a biofilm on the stainless steel coupons after growth for 3 days in marine broth with cell densities above 6.2 log CFU/cm^2. *Vibrio* strain B37bio formed a thin layer, with only 4.8 log CFU/cm^2. The highest cell density in the 3-day-old biofilms was observed for all *P. tunicata* strains independently of color and for *Phaeobacter* strains (Table 5). When marine broth-coated steel plates were submerged in a suspension of the biofouler *Pseudoalteromonas* S91, it attached at a level of 5.9 log CFU/cm^2 after 24 h.

In particular, strains of the four *Pseudoalteromonas* species *P. piscicida*, *P. tunicate*, *P. ulvae*, and *P. aliena* had a strong antiadhesive effect. All strains of these species significantly reduced the numbers of attaching *Pseudoalteromonas* strain S91 cells to below or equal to the detection limit (1.6 log CFU/cm^2) (Fig. 2a). Two different patterns were observed for the antiadhesive effect. The *P. piscicida* strains were bactericidal, since no S91 bacteria could be detected in the suspension surrounding the coupons after 1 (data not shown), 4 (Table 5), or 24 h (Fig. 2b). The *P. tunicate*, *P. ulvae*, and *P. aliena* strains only reduced the number of S91 in the suspension with 1 to 2 log CFU/ml independently of the pigmentation of the strains (Table 5). This level of growth inhibition equals the inhibition observed for the other strains.

The *Phaeobacter* strains, including the control *Phaeobacter* strain 27-4, reduced the number of adhered S91 cells by approximately 1 log unit, and it may be linked to a bactericidal

TABLE 5. Biofilm formation by marine antibacterial strains and their killing and antiadhesive effect against *Pseudoalteromonas* S91 and antisettlement effect against *Ulva australis*

Species and strain	Pigmentation	Biofilm formation on stainless steel[a]	Biofilm formation on plastic[b]	Reduced no. of adhered S91 cells[c]	Reduced no. of S91 cells in suspension[d]	Reduced no. of settled *Ulva* zoospores[e]
P. piscicida						
A23a-4a	Yellow	++	+	+++	+++	+++
A38q-4a	Yellow	++	+	+++	+++	+++
B39bio	Yellow	++	+++	+++	+++	++
P. tunicata						
J49q-3a	Purple	+++	++	+++	+	+++
J36q-4a	Yellow	+++	+	+++	++	+++
J38a-5a	Yellow	+++	+	+++	+	+++
P. ulvae						
H34q-5a	Purple	+++	+++	+++	+	++
A24a-4a	Purple	++	+++	+++	+	++
P. agarivorans						
F55bio	Beige	++	++	+	+	+++
P. aliena						
F51a-1b	White	++	++	+++	+	++
P. antarctica						
B54q-3a	Black	++	+	+	+	+++
E45q-4a	Brown	++	+	+	+	−
Vibrio sp.						
A31bio	White	++	+++	−	+	−
A32bio	White	++	++	−	−	+
B37bio	White	+	+	−	−	−
Photobacterium sp.						
K29bio	Light yellow	++	+	−	+	−
J46bio	White	++	+	−	−	−
H38bio	White	++	+	+	+	+++
I46bio	White	++	+	++	+	−
Phaeobacter sp.						
A36a-5a	Brown	+++	+++	+	++	−
A49a-4a	Brown	++	+++	+	++	−
A40a-4a	Brown	++	+++	+	++	−
Controls						
P. tunicata						
D2	Purple	+++	++	+++	+	+++
Phaeobacter						
27-4	Brown	+++	+++	+	++	−

[a] The amount of bacterial cells was quantified by plate count on marine agar after removal by ultrasonication. +, <6 log CFU/cm^2; ++, between 6 and 7 log CFU/cm^2; +++, >7 log CFU/cm^2.
[b] The amount of biofilm after crystal violet staining, measured at the OD_{550}. +, OD_{550} of <0.5; ++, OD_{550} between 0.5 and 1.5; +++, OD_{550} of >1.5.
[c] Log reduction (CFU/cm^2) of adhered *Pseudoalteromonas* S91 after 4 h of incubation compared to that on marine broth-coated surfaces. −, <0.5 log reduction; +, between 0.5 and 1.5 log reduction; ++, between 1.5 and 3 log reduction; +++, >3 log reduction.
[d] Killing of *Pseudoalteromonas* S91 after 4 h of incubation. −, <0.5 log reduction; +, between 0.5 and 1.5 log reduction; ++, between 1.5 and 3.0 log reduction; +++, >3 log reduction.
[e] Settlement of *Ulva australis* spores on bacterial biofilm compared to that on marine broth-coated surfaces. −, >90% settlement; +, between 50 and 90% settlement; ++, between 10 and 50% settlement; +++, <10% settlement.

effect, since numbers of S91 were reduced with approximately 2 log CFU/ml in the suspension surrounding the coupons.

The *Photobacterium* strain I46bio reduced the number of adhered S91 cells by almost 2 log units, which differed from the other strains in the *Vibrionaceae* group that did not have any or had only a slight reducing effect (Table 5). The bactericidal or bacteriostatic effect of the *Vibrionaceae* against S91 was less than 1.5 log CFU/ml.

Anti-*Ulva* spore settlement and germination activity of marine antibacterial bacteria. Anti-*Ulva* spore activity was tested by exposing spores directly to monoculture biofilms. All 22 bacteria formed biofilms on the surface of the microtiter wells as measured by crystal violet staining (Table 5).

Twelve of the 22 strains were able to reduce the number of settling *Ulva* spores to below 10% of the level on control surfaces coated with marine broth (Table 5). All except one of these strains belonged to the *Pseudoalteromonas* group (Table 5). Of the 12 *Pseudoalteromonas* strains tested, only one (identified as *P. antarctica*) was unable to inhibit the settlement of *Ulva* spores, indicating a strong effect on zoospore attachment in this genus. The inhibitory effect against *Ulva* spores varied between strains belonging to the *Vibrionaceae* group (Table 5). One *Vibrio* strain reduced the number of settled spores slightly; one *Photobacterium* strain reduced the number of spores to 8%, whereas three strains enhanced the settlement of spores. None of three *Phaeobacter* strains inhibited *Ulva* spore settlement, and two of the strains did in fact mildly stimulate the settlement (data not shown).

The inhibitory effect on the germination of *Ulva* spores by bacterial biofilms was tested after incubation for 7 days. There was no correlation between the number of attached *Ulva* spores and the number of these that actually germinated (Fig. 3). The presence of marine broth on the surface did not change the number of spores that settled (data not shown) but en-

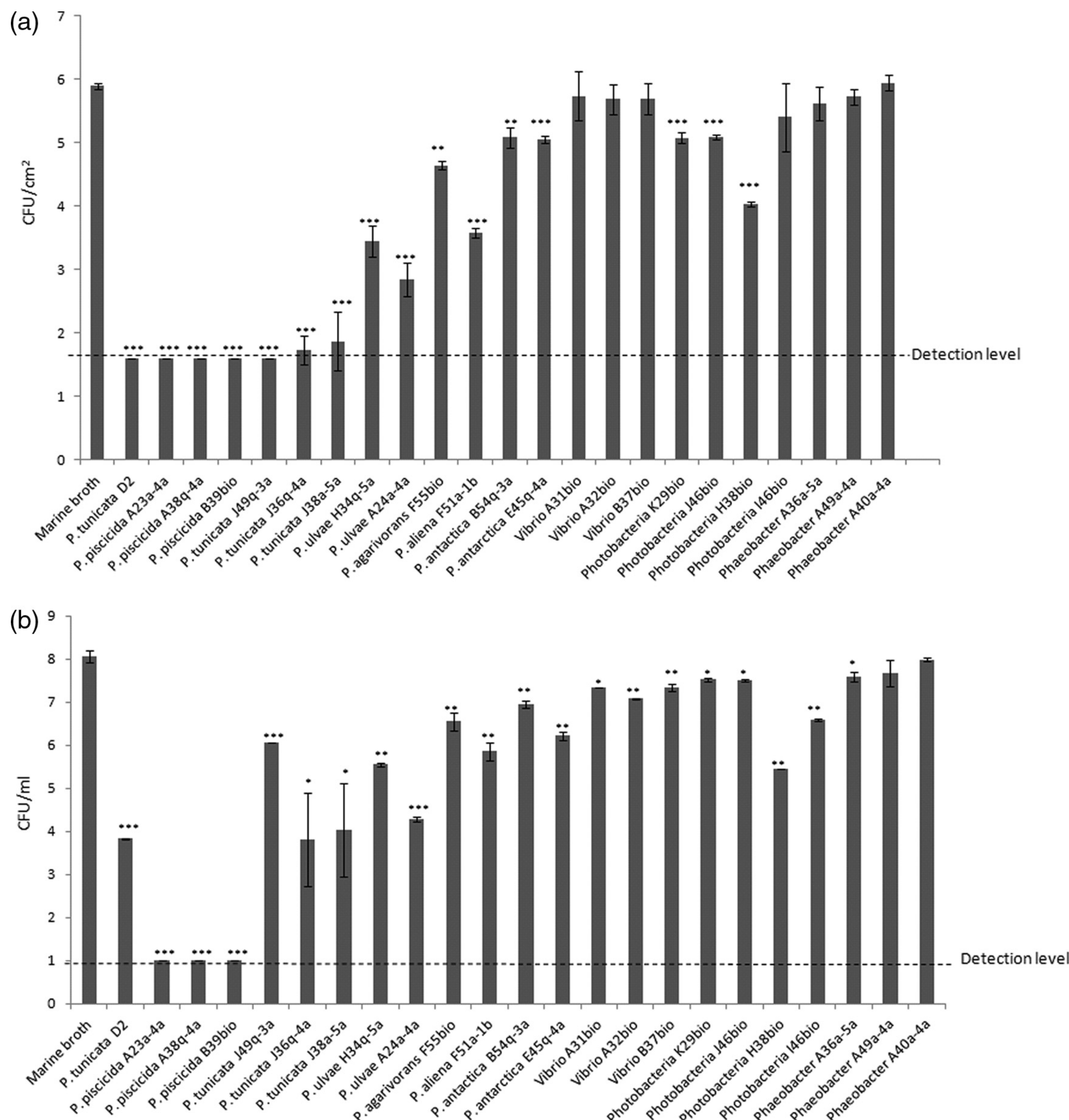

FIG. 2. Number of *Pseudoalteromonas* S91 organisms (a) attached to stainless steel (CFU/cm^2) precoated with different marine bacteria and (b) in the surrounding suspension (CFU/ml) after 24 h. *, **, and *** indicate that the number of *Pseudoalteromonas* S91 cells adhered to stainless steel and in the suspension is significantly different from that of the control (marine broth) at 5, 1, and 0.1% levels, respectively.

hanced the number of spores germinating (Fig. 3). The germination of the settled spores depended on the bacterial biofilm. Spores that attached to a biofilm of *Pseudoalteromonas* elongated and had a wide diameter compared to that of attached spores on a *Vibrio* biofilm, which did not germinate within 7 days of incubation (Fig. 3).

DISCUSSION

Bacteria from marine environments represent a relatively untapped resource of bioactive compounds (19, 31, 33). In this study, we isolated bacteria with antibacterial and antifouling activity (summarized as bioactive bacteria) within a relatively small geographic area (the coastline of Denmark). Bioactive bacteria predominantly belonged to the *Vibrionaceae* family, *Pseudoalteromonas*, and the *Roseobacter* clade, and the isolation strategy (Table 2) influenced the type of genera being isolated. The screening for bioactive bacteria during this 1-year study at 11 coastal sites of Denmark demonstrated that numbers and types of bioactive bacteria varied with both season and niche. This indicates that both temporal and spatial screening is important if different bioactive bacteria are to be isolated.

Our prime purpose was not the identification of antibacterial activity *per se* but the detection of additional antifouling activity expressed by these bacteria. Antibacterial activity could not, however, directly serve as a proxy for broader antifouling effect as measured against bacterial or algal spore attachment. Twenty-

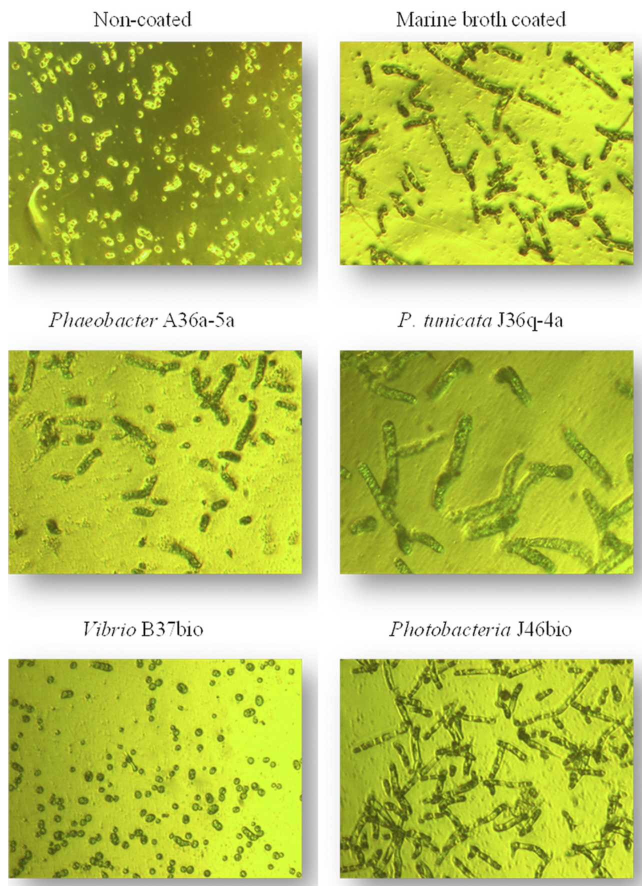

FIG. 3. Germination of *Ulva* on bacterial biofilms (40× magnification).

two of the marine bacteria that inhibited the growth of *V. anguillarum* were tested for their ability to prevent the adhesion of *Pseudoalteromonas* strain S91, and *Pseudoalteromonas* species were particularly effective in preventing bacterial adhesion. *P. piscicida*, *P. tunicata*, *P. ulvae*, and *P. aliena* prevented any adhesion of *Pseudoalteromonas* S91 by two distinct mechanisms. While *P. piscicida* had strong bactericidal activity, *P. tunicata* (including strain D2), *P. ulvae*, and *P. aliena* were not bactericidal against S91 but prevented the adhesion. In the agar-screening (replica) plating, *P. piscicida* strains consistently caused the largest clearing zones, which could indicate that they produce an antibacterial compound more potent and diffusible than those of the other *Pseudoalteromonas* species, thus explaining the rapid killing of S91 in suspension. We speculate that the non-piscicida *Pseudoalteromonas* strains produce a specific antiadhesive molecule(s). Such a compound(s) has been found in other *Pseudoalteromonas* strains. In strain 3J6, a proteinaceous substance reduced attachment by other bacteria (10). Further, the strains may produce exopolysaccharides (EPS) containing galactosamine, which reduce the adhesion of marine bacteria which use the autoinducer type-2 signaling system (30). Several *Pseudoalteromonas* species contain galactosamine in their EPS layer (18), and the target strain may use the type 2 signaling system, as previously shown for other *Pseudoalteromonas* strains (5). The antifouling *Pseudoalteromonas* also may produce quorum-sensing inhibitors or biosurfactants (10). Further studies clearly are required to elucidate the mechanism behind the nonbactericidal antifouling effect.

Pseudoalteromonas strains caused more pronounced inhibitory effects in the algal spore settlement assay than strains belonging to the *Vibrionaceae* and *Phaeobacter*. This parallels a study (34) in which spores of the green algae *Enteromorpha* were more efficiently repelled by *Pseudoalteromonas* strains than *Vibrio* spp. We did not observe any correlation between bactericidal effect and the prevention of *Ulva* zoospore settlement.

The presence of pigmentation in *Pseudoalteromonas* species has been linked to their antifouling capacity, as expressed by their ability to reduce algal spore and larval settlement (13, 14, 22, 26). Nonpigmented mutants of *P. tunicata* D2 (14) or of the red *Pseudoalteromonas* strain sf57 (26) lost the antisettlement activity seen in the purple/red pigmented wild types, and Holmström et al. (22) also suggested that pigmentation and the antisettlement effect are correlated, as they isolated two white *Pseudoalteromonas* strains without anti-*Ulva* activity and eight pigmented strains with activity. However, our results indicate that mechanisms other than pigmentation can be responsible for antisettlement activity in *Pseudoalteromonas*, since our two white/beige strains (*P. aliena* and *P. agarivorans*) reduced the settlement of *Ulva* spores, whereas the brown *P. antarctica* did not.

The antibacterial protein AlpP has been suggested to be the mediator for the anti-adhesive activity and bactericidal effect, particularly in *P. tunicata* D2 (28, 32, 38, 39), and may be a useful proxy in screening studies. All four *P. tunicata* strains harbored the gene coding for AlpP. However, the *alpP* gene was not detected in other *Pseudoalteromonas* strains with antifouling ability (42), indicating that either another compound(s) is responsible for the observed antifouling effect or that the *alpP* primers are too specific, as they are constructed from only one known *P. tunicata* sequence (42). One could speculate that a similar mechanism is used by all three bacteria, since they are genetically similar at least on the 16S rRNA gene sequence level (Fig. 1) and since we could not link the antifouling activity to pigmentation as previously suggested (14). *P. aliena* is nonpigmented, whereas two *P. tunicata* strains were bright yellow and one *P. tunicata* strain and the *P. ulvae* strains were purple.

Another relevant feature of antifouling activity is the ability of a bacterial biofilm to prevent the germination and growth of the zoospores already attached. A study using supernatants from a *Pseudomonas* sp. and different *Bacillus* spp. suggested that the anti-settlement effect is not necessarily related to the prevention of germination and growth of *Ulva* zoospores. A *Pseudomonas* strain was able to prevent both settlement and germination, whereas *Bacillus licheniformis* only reduced settlement (7). The germination of already settled zoospores on bacterial biofilms was, in the present study, related to the bacterial genus, as all *Pseudoalteromonas* and *Phaeobacter* strains resulted in large germinated spores, whereas the zoospores attached to *Vibrio* biofilm did not germinate at all independently of the number of spores settled (Fig. 3). Part of the difference in the germination may be linked to different amounts of nutrient left for the zoospores, since we observed a lack of germination on noncoated surfaces, whereas marine

broth-coated surfaces resulted in germination to slime algae. The germination on the marine broth-coated surface also may be a result of a bacterial biofilm being developed over time, since zoospores are not sterilized prior to use. It may, however, also be due to bioactive compounds being produced by the marine bacteria, and further studies are required to verify this hypothesis.

The *Phaeobacter* strains inhibited the growth of both *V. anguillarum* and *Pseudoalteromonas* S91, but interestingly they facilitated spore settlement to their bacterial biofilms. We tested the *Phaeobacter* strains in a monitor assay for acylated homoserine lactones (AHLs) (*Agrobacterium tumefaciens*) and all were positive (data not shown), indicating that AHL production could be the cue in promoting algal settlement by these strains as well. Rao et al. (38) similarly observed that high cell densities of *Phaeobacter* sp. strain 2.10 attracted *Ulva* sp. spores (38), and the production of the density-sensing signal molecules of acyl homoserine lactones (AHLs) has been suggested previously to act as a settlement cue for *Ulva* spores in general (44).

We observed a significant difference in the number of bioactive bacteria isolated in different months, with the largest numbers being observed in August and November. This may be an artifact of the isolation method used (incubation at 20°C), but we continuously observed large variations within the *Roseobacter* clade bacteria and the *Pseudoalteromonas* genus during the year in particular, indicating a season-specific marine microbiota. This was most profound for bacteria belonging to the *Roseobacter* clade, which all were isolated from one location and only when the water was visually green and turbid, indicating phytoplankton blooms. *Roseobacter* clade bacteria often are associated with algal blooms, where they can constitute more than 20% of the prokaryotic DNA (17); however, we did not isolate *Phaeobacter* from the alga-containing water samples but did isolate them from heavily fouled surfaces in Jyllinge harbor, indicating that they used algal components but were specifically associated with the fouling macroalgal community. Cooccurrence between such fouling and bacteria within the *Roseobacter* clade has been described and suggested to be related to the need for cell-surface interaction for some lineage members in this clade (43).

Bioactive *Pseudoalteromonas* species were isolated year-round; however, there was a seasonal pattern in the *Pseudoalteromonas* species isolation, with *P. arctica*, *P. antarctica*, and *P. prydzensis* occurring predominantly in the colder months. It is well known that several pigmented *Pseudoalteromonas* species are antibacterial (6), hence it is not surprising that these were isolated in our sampling because we deliberately included pigmented colonies when testing for antibacterial activity against *V. anguillarum*. In contrast to a recent global sampling for antibacterial bacteria, we did not isolate *P. luteoviolacea* and *P. rubra* (19, 47), which is likely explained by their preference for tropical waters (47). This indicates that temperature is an important factor influencing the species composition of the antibacterial *Pseudoalteromonas* population. However, local adaptation to specific econiches also was found. *P. tunicata* was isolated from only 2 of 11 locations, and both were heavily fouled with algae and sea grass. The *P. tunicata* strains all were surface associated, indicating a preference of this bacterium to colonize surfaces, which corresponds to results from previous isolation sites (15, 23) and to genome analysis revealing properties of a surface-associated lifestyle (46).

The battle against fouling is ongoing, and the search for antifouling compounds or principles that can reduce or eliminate the attachment of marine organisms is intense (2, 37). While several studies have demonstrated that specific marine bacteria produce compounds with antifouling activity against a range of marine micro- and macroorganisms (15, 48), our study is the first to elucidate the antifouling potential of a range of antibacterial bacteria from different niches and seasons. We demonstrated that several groups of antibacterial bacteria could be isolated even within a relatively small geographic region, but that both season and local parameters influenced the genus or species of bacteria isolated. Our study further indicated that antifouling activity against both a marine fouling bacteria and against *Ulva* zoospores were predominantly observed within the antibacterial *Pseudoalteromonas* group. Interestingly, two different mechanisms caused the antibacterial fouling effect of *Pseudoalteromonas*. One was likely caused by the bactericidal activity of *P. piscicida*, whereas the other was not dependent on bactericidal activity. This observation is relevant if marine bacteria or their bioactive compounds are to be used as antifouling agents, since the antifouling effect caused by bactericidal activity may result in the build-up of microbial resistance. Hence, elucidating the antifouling principle of *P. tunicata*, *P. ulvae*, and *P. aliena* may result in the identification of novel antifouling compounds which can prevent fouling without killing marine microorganisms.

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