Ecology, Inhibitory Activity, and Morphogenesis of a Marine Antagonistic Bacterium Belonging to the *Roseobacter* Clade

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Roseobacter strain 27-4 has been isolated from a turbot larval rearing unit and is capable of reducing mortality in turbot egg yolk sac larvae. Here, we demonstrate that the supernatant of *Roseobacter* 27-4 is lethal to the larval pathogens *Vibrio anguillarum* and *Vibrio splendidus* in a buffer system and inhibited their growth in marine broth. Liquid chromatography (LC) with both UV spectral detection and high-resolution mass spectrometry (HR-MS) identified the known antibacterial compound thiotropocin or its closely related precursor tropodithietic acid in the bioactive fractions. Antibacterial activity correlated with the appearance of a brownish pigment and was only formed in marine broth under static growth conditions. A thick biofilm of multicellular star-shaped aggregated cells formed at the air-liquid interface under static growth conditions. Here, the bioactive compound was the base peak in the LC-UV chromatograms of the extracts where it constituted 15% of the total peak area. Aerated conditions results in 10-fold-higher cell yield, however, cultures were nonpigmented, did not produce antibacterial activity, and grew as single cells. Production of antibacterial compounds may be quorum regulated, and we identified the acylated homoserine lactone (3-hydroxy-decanoyl homoserine lactone) from cultures of *Roseobacter* 27-4 using LC-HR-MS. The signal molecule was primarily detected in stagnant cultures. *Roseobacter* 27-4 grew between 10 and 30°C but died rapidly at 37°C. Also, the antibacterial compounds was sensitive to heat and was inactivated at 37°C in less than 2 days and at 25°C in 8 days. Using *Roseobacter* 27-4 as a probiotic culture will require that is be established in stagnant or adhered conditions and, due to the temperature sensitivity of the active compound, constant production must be ensured.

The dramatic growth in the aquaculture sector (an increment of 9 to 10% per year over the last 8 to 10 years) has emphasized the importance of fish disease control. Bacterial diseases are important constraints and may be treated with antibiotics. However, due to the risk of development and transfer of antibiotic resistance, alternative disease control measures must be implemented. Vaccines have been very successful also in fish farming; however, vaccines are not efficient at the larval stages. Several studies have demonstrated that probiotic bacteria may be used to control pathogenic organisms in fish larval rearing (17, 18, 32), and one of several promising candidates are bacteria of the marine *Roseobacter* clade.

The ability of *Roseobacter* to inhibit other bacteria was noted already by Ruiz-Ponte et al. (31) who used *Roseobacter gallaeciensis* strain BS107 as a probiotic treatment of scallop larvae (32). *Roseobacter* strains have also been isolated from turbot larval farms, and they were selected from this environment due to their strong anti-*Vibrio* activity (18). These strains appeared to constitute a relatively stable community since the same

DNA subtypes were isolated over several months (19). Antagonistic *Roseobacter* were especially associated with the walls of the rearing tanks, and this probably reflects the fact that *Roseobacter* strains are excellent biofilm-forming organisms and are among the first and dominant colonizers of surfaces in marine environments (9).

The genus *Roseobacter* and species affiliated with this group (the *Roseobacter* clade) are important members of the marine microbiota (14, 33). These aerobic organisms, which may account for as much as 40% of prokaryotic DNA from the ocean, are believed to play an important role in the sulfur cycle of the oceans (27). Several *Roseobacter* species are inhibitory toward other bacteria and Brinkhoff et al. (3) recently demonstrated that the inhibitory compound produced by the *Roseobacter* strain T5 is the sulfur containing tropodithietic acid. This is a precursor of the antibacterial compound thiotropocin isolated from a marine isolate of *Agrobacterium* (37).

Roseobacter are typically associated with algae and dinoflagellates (12, 14, 26), and they may therefore constitute a very suitable probiotic in the rearing of marine fish larvae. Algae are used as live feed for the rotifers and *Artemia*, which are used as live feed for marine fish larvae. Hence, the algae may be used as a vehicle for the probiotic bacteria.

To facilitate and optimize the use of a probiotic bacterium,

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it is necessary to understand its ecology, especially how growth conditions and environmental factors influence the antibacterial activity. The production of antibacterial compounds may in some strains be controlled in a quorum-dependent manner (2, 36, 42). The quorum size of the bacterial population is sensed by monitoring the concentration of a signal compound, which the bacteria release into the environment (10, 11, 40). In gram-negative bacteria, these compounds are mainly acylated homoserine lactones (AHLs) and, indeed, *Roseobacter* produce compounds that induce AHL monitor systems (4, 15, 22). We therefore hypothesized that production of the antibacterial compound of *Roseobacter* could be AHL regulated.

The present study was undertaken with the purpose of determining the parameters influencing growth and antibacterial activity of a *Roseobacter* strain suitable for disease control in turbot larval rearing systems.

MATERIALS AND METHODS

Bacterial strains and media. *Roseobacter* strain 27-4 was isolated from turbot larval rearing units due to its inhibitory activity against turbot larval pathogenic bacteria (18). In brief, samples from turbot rearing units were plated on marine agar (MA Difco 212185) and subsequently replica plated on an agar cast with *Vibrio anguillarum* strain 90-11-287 (serotype O1 strain [35]. Inhibitory activity of strains causing "clear zones" in this agar was confirmed against *Vibrio anguillarum* and *Vibrio splendidus* strain DMC-1 (18; R. Thomson, H. L. Macpherson, A. Riaza, and T. H. Birkbeck, unpublished data). *Pseudomonas fluorescens* strain AH2 (16) was used as a positive inhibition control in the well diffusion assays. All strains were stored at −80°C in medium rich in glycerol and dried skim milk (13). *Roseobacter* strains were identified based on phenotypic tests and 16S rRNA gene sequence analysis (18). All strains were grown in marine broth (MB; Difco 279110) or on marine agar. *Roseobacter* strain 27-4 was isolated from rotifer feeding on algal.

Assessment of antibacterial activity in a well diffusion assay. Antibacterial activity was measured in a well diffusion assay using *V. anguillarum* as the target organism (18). The level of antibacterial activity was determined by using a semiquantitative assay. Sterile-filtered 27-4 supernatants were twofold serially diluted in sterile-filtered MB. A total of 60 μl of each dilution was tested in the assay, and activity was expressed in arbitrary units (the reciprocal of the highest dilution causing an inhibition zone).

Effect of 27-4 antibacterial "compound" on *V. anguillarum* and *V. splendidus*. (i) Inactivation. *V. anguillarum* 90-11-287 and *V. splendidus* DMC-1 were precultured in MB at 20°C for 1 to 2 days and reinoculated in MB. Bacteria were harvested after 24 h at 2,500 × *g* for 5 min, resuspended, and diluted in phosphate-buffered saline (5× PBS; 19.8 g of Na₂HPO₄·2H₂O, 5.6 g of NaH₂PO₄·1H₂O, and 36.6 g of NaCl to 1 liter [pH 7.2]) to 10^4 to 10^5 CFU/ml. *Vibrio* suspensions were mixed with 20, 50, or 80% of supernatants from *Roseobacter* grown under stagnant conditions in marine broth at 20°C. All vials were incubated at 20°C and *Vibrio* counts determined at regular intervals by plating on MA.

(ii) Inhibition of growth. Sterile-filtered 27-4 supernatant (produced as described above) was twofold diluted with sterile-filtered MB (to remove insoluble salts) and pipetted into microtiter plates (TV Microwell p6U; Nunc, Roskilde, Denmark). *V. anguillarum* and *V. splendidus* were grown in sterile-filtered MB at 20°C and inoculated at an initial cell density of ca. 10^2 CFU/ml in the MB-supernatant mixtures. Concentrations of supernatant were 90, 45, 23, 11.3, 5.6, and 2.8%. Plates were incubated at 20°C and growth, followed by determination of the absorbance at 450 nm (Versamax; Molecular Devices).

Conditions influencing growth, antibacterial activity, and pigmentation of *Roseobacter* strain 27-4. *Roseobacter* strain 27-4 was grown in MB at 20 to 25°C until brown pigment was visible (3 to 7 days) and reinoculated in MB. This medium was also used for temperature and aeration experiments. Experiments were conducted at stagnant and aerated (200 rpm) conditions at 20 to 25°C. The influence of salts on growth and activity was investigated in 2 and 3% Instant Ocean salts (IO; Aquarium Systems, Inc., Sarrebourg, France). All experiments were conducted in the dark. Screening for AHLs was done on sterile filtered culture supernatants using the AHL monitor bacteria *Agrobacterium tumefaciens* NT1(pZLR4) (7) and *Chromobacterium violaceum* CV026 (25, 36) in a well diffusion assay (29). Since pH increased in aerated cultures to 8.4 and AHLs are

unstable at high pH, the MB was buffered with 0.3 M HEPES (H-3375; Sigma) and adjusted to 7.0 to avoid increasing the pH during culturing.

Growth of strain 27-4 was determined by spread plating on MA with concomitant sampling for antibacterial activity, i.e., sterile-filtered supernatants, which were kept at −20°C until analysis in well diffusion assay. Pigment was measured by spectroscopy in sterile-filtered supernatant at 398 nm (UV maximum of the pigment).

Synthesis of *N*-(3-hydroxydecanoyl)-homoserine lactone. Ethyl 3-oxodecanoate was synthesized by reaction of octanal with ethyl diazoacetate in the presence of SnCl₄. This compound was then reduced with sodium tetraborohydride in ethanol, affording ethyl 3-hydroxydecanoate, which was saponified with potassium hydroxide in methanol. The free acid was reacted with L-homoserine lactone hydrobromide after activation with benzotriazol and dicyclohexylcarbodiimde. The product was purified by chromatography on silica. The product was checked by using nuclear magnetic resonance, gas chromatography, and liquid chromatography combined with photodiode array detection (UV) and high-resolution mass spectrometry (HR-MS; both positive and negative electrospray) as described below, showing that *N*-(3-hydroxydecanoyl)-homoserine lactone was more than 98% pure.

Scanning electron microscopy. *Roseobacter* strain 27-4 grown in MB at 20 to 25°C for 3 to 5 days was prepared by two different methods. In the first (see Fig. 3a), a small drop from the surface layer was placed on a circular glass slide (diameter, 5 mm) and, after the cells were allowed to settle for 5 min, the medium was carefully removed by using filter paper and exposed to osmium vapor for 1 h. Subsequently, the cells were coated with gold-palladium and observed in a Quanta 200 SEM at 20 kV. In the second method (Fig. 3b), 20 to 40 μl of surface layer suspension was carefully filtered through a polycarbonate filter (pore size, 0.2 μm). In the subsequent fixation and dehydration procedure the filters were placed floating on the surface of the liquids with the bacteria on the upper side. The procedure used was fixation in glutaraldehyde and osmium tetroxide, followed by dehydration in a graded series of ethanol and final drying in hexamethyldisilazan. The samples were mounted on aluminum stubs and sputter coated as in the first method.

Physical and chemical characteristics of *Roseobacter* antibacterial compound. *Roseobacter* strain 27-4 was grown in MB for 3 to 7 days at 20°C, reinoculated in MB, and grown for 2 to 3 days. Supernatants from outgrown cultures were sterile filtered.

(i) Temperature stability. Aliquots of 1 ml of sterile-filtered culture supernatant were incubated at 5, 20, 25, 30, and 37°C for 30 days. Long-term stability at freezing (−80 and −20°C) was tested for up to 7 months. At regular intervals, samples were tested in agar diffusion assay for antibacterial activity against *V. anguillarum*. Thawed tubes were discarded. At 100°C aliquots of 1.2 ml were boiled for 12 min before we tested for antibacterial activity.

(ii) Effect of NaCl and pH. Aliquots (5 ml) of sterile-filtered supernatant were adjusted to pH 1, 3, 5, 7, or 9 with 0.2 or 2 M HCl or NaOH. Sodium chloride was added to a final concentration of 5, 10, 15, or 30% (wt/vol). All suspensions were placed at room temperature for 1 to 1.5 h, and activity was tested in the agar diffusion assay.

(iii) Purification of compound. *Roseobacter* 27-4 was grown in 500 ml of MB in a 5-liter volumetric flask at 25°C for 4 days. The cells were removed by centrifugation (10,000 × *g* for 10 min), and the pH of the supernatant was adjusted to 3.5, followed by extraction with 500 ml of ethyl acetate acidified with 0.1% formic acid (FA) repeated three times, which after phase separation was transferred to a vessel and evaporated to dryness under nitrogen flow. The dry ethyl acetate extract was redissolved in 9 ml of acetonitrile (ACN)-water (1:19) containing 1% FA, and these sequentially applied to two 60-mg Oasis MAX columns (Waters, Milford, MA) coupled in series. These had previously been sequentially conditioned with 4 ml of methanol (high-pressure liquid chromatography grade) and 3 ml of ACN-water (1:19) containing 1% FA. After the samples were loaded by gravity, the columns were washed with 4 ml of PBS buffer (pH 7). Then, 3.5 ml of ACN-water (1:1) was passed through the column and collected (fraction 1), followed by 3.5 ml of ACN-water (9:1; fraction 2), 3.5 ml of ACN-water (1:1) with 2% FA (fraction 3), and finally 3.5 ml of ACN-water (9:1) with 2% FA (fraction 4). The solvents were then removed in vacuo on a SpeedVac (ThermoSavant, Holbrook, NY).

(iv) LC-UV-HR-MS analysis. Extracts were redissolved in ACN-water (1:1), filtered through a 0.45-μm-pore-size PFTE syringe filter and analyzed by reversed-phase chromatography combined with photodiode array detection (UV) and HR-MS (LC-UV-HR-MS) on an LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom). Samples were separated on a Luna C₁₈ (II) by using an acetonitrile-water gradient system and analyzed in the positive electrospray ionization (ESI⁺) as described previously (5). The only changes were that two scan functions (1 s each) were used:
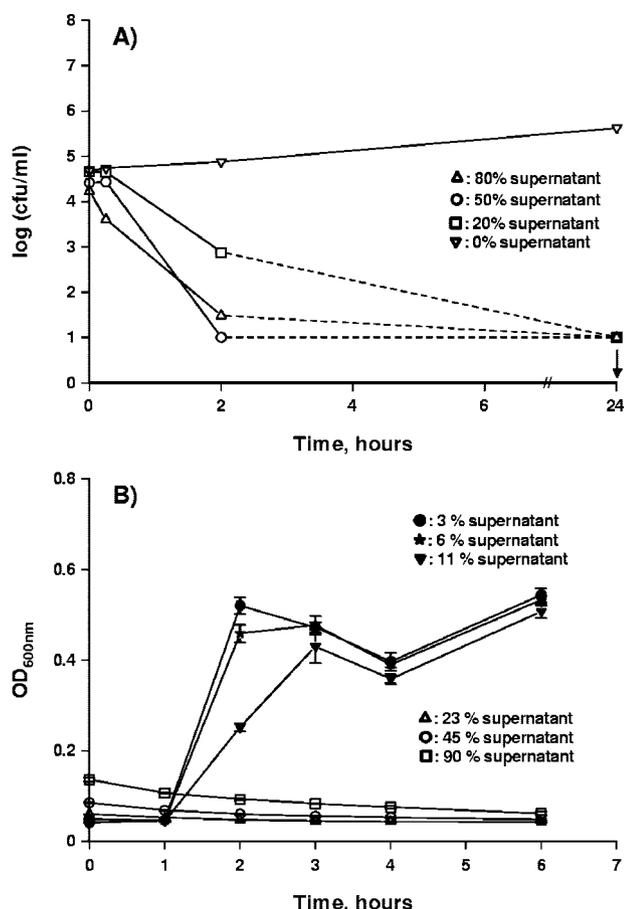
FIG. 1. Survival (A) or growth (B) of *V. anguillarum* when exposed to sterile-filtered supernatant from *Roseobacter* strain 27-4. Strains were suspended in buffer and incubated at 20°C. The datum points are means of duplicates, and error bars indicate standard variations.

one with a potential difference of 10 V between the skimmers and one with 30 V (to induce in-source fragmentation). Samples were also analyzed in the negative electrospray ionization (ESI⁻) mode by using the same LC-UV-HR-MS system. Here, the acetonitrile-water gradient system started 15% acetonitrile, which was increased linearly to 100% in 20 min, holding this for 5 min. Formic acid at 200 μl/liter was added to the water. The MS was tuned to a resolution of 5,000 (at half peak height), and two scan functions (1 s each) were used: one with a potential difference of 10 V between the skimmers and one with 30 V. The capillary was held at −1,800 V, and data were collected as centroid data from *m/z* 100 to 900. A solution of 3,4-dihydroxybenzoic acid in water-methanol (1:1 [vol/vol]) was infused (10 μl/min) into the lock spray source (second ESI⁻ spray) by using a syringe pump. The [M-H]⁻ ion of 3,4-dihydroxybenzoic acid was subsequently used for online mass correction every 3 s.

RESULTS

Effect of antibacterial compound on *V. anguillarum* and *V. splendidus*. Cells of *V. anguillarum* or *V. splendidus* were killed when exposed to sterile-filtered supernatants of *Roseobacter* strain 27-4 (Fig. 1A). *V. anguillarum* cells were reduced with 3 log units when exposed to 50 to 80% supernatant for 2 h. *V. splendidus* was less sensitive, and the same volume of *Roseobacter* supernatant caused an approximately 1 log unit reduction in cell count. None of the *Vibrio* spp. were detected after 24 h (<10 CFU/ml).

Autoinhibition was tested by exposing *Roseobacter* cells to *Roseobacter* supernatant. Cells were harvested prior to station-

ary phase and diluted 10 times, and 10⁷ CFU/ml were mixed with 80% supernatant. No reduction of *Roseobacter* cells was observed after 24 h.

V. anguillarum and *V. splendidus* were exposed to *Roseobacter* sterile-filtered supernatant mixed in MB, and growth of *V. anguillarum* was completely inhibited by addition of 23 to 90% supernatant (Fig. 1B). *V. splendidus* was less sensitive and grew also with the addition of 23% supernatant.

Factors influencing production of antibacterial compound. Cultures of *Roseobacter* grown in MB were always characterized by the production of a brownish diffusible pigment when antibacterial activity could be detected. However, this pigmentation was only produced under some cultures conditions.

(i) Aeration. When *Roseobacter* 27-4 was grown in MB under static conditions, pigment production always started at the liquid-air interface where a biofilm was formed. The surface-volume ratio influenced pigmentation and the level of antibacterial activity (Table 1). *Roseobacter* 27-4 grew well under aerated conditions and increased from 10⁴ CFU/ml to approximately 10¹⁰ CFU/ml in 24 h, whereas the static grown culture ceased at a cell density of approximately 10⁹ CFU/ml within 48 h. Antibacterial activity, pigmentation, and biofilm formation was only seen under static growth conditions (Fig. 2). Also, the morphology of the cultures differed dramatically between the two growth conditions. In the static cultures, the bacteria did not grow as individual cells but in a multicellular star-forming shape, and the "stars" merged to form a thick biofilm (Fig. 2a). In contrast, the aerated culture was dominated by single, individual cells (Fig. 2b). When observed in a phase-contrast microscope (×1,000 magnification), the cells seem to divide into the star shape rather than single cells attaching to each other to for the star shape. Furthermore, no stars were formed when incubating a full grown aerated culture under static conditions. The scanning electron microscopy of the culture stained directly revealed a clear star-shaped aggregate where salt crystals and a layer of slime covering the cells was visible (Fig. 3a). When the cells were fixed and washed in buffer, salt crystals and slime were removed, and the aggregation of four to ten cells in star-shaped structures was clearly revealed (Fig. 3b). The individual cells were anchored to one another with fibrils.

The star-shaped structure was not stable since vigorous shaking broke up the star shape into individual cells. There-

TABLE 1. Antibacterial activity and pigmentation of *Roseobacter* strain 27-4 grown in MB at 20°C under static conditions

Vol of MB in 400-ml flasks	Pigment, ABS 398 nm	Anti-*Vibrio* activity[a]	
		Diam in well diffusion assay (mm)	Arbitrary units
25	1.82	29	512
50	1.40	27	256
100	0.97	22	64
150	0.87	22	128
200	0.73	19	32
250	0.48	15	16
300	0.24	9	8
350	0.14	8	2

[a] Activity was measured as arbitrary units denoting the reciprocal of the highest twofold dilution causing inhibition of the target organism.
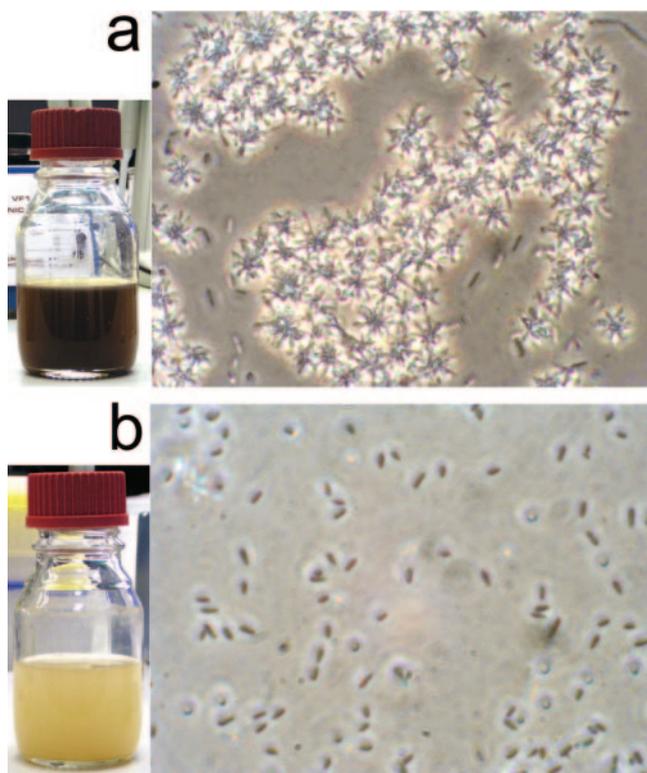
FIG. 2. *Roseobacter* strain 27-4 grown in MB at 25°C under static and aerated conditions. Pictures are from phase contrast microscope at ×1,000 magnification. One bacterial cell is approximately 1 by 3 μm.
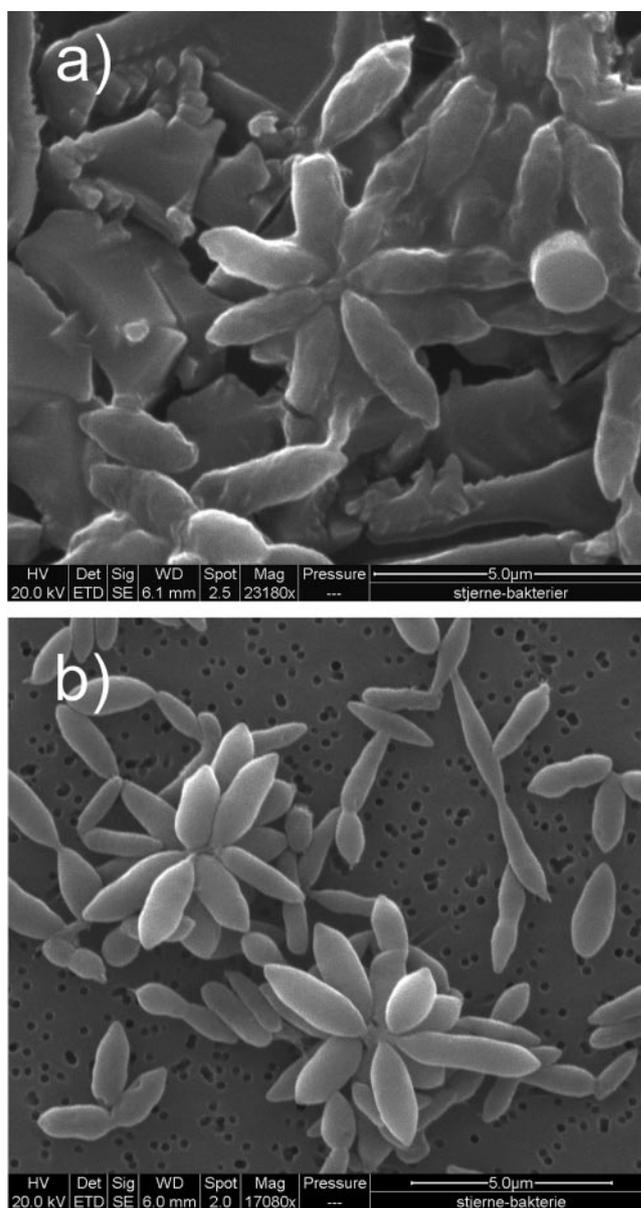

FIG. 3. Scanning electron micrographs of *Roseobacter* strain 27-4 grown in MB under stagnant conditions. (a) Cells were only exposed to osmium vapor and subsequently coated with gold-palladium. (b) Standard fixation and dehydration procedure was used (see Materials and Methods).

fore, plate counting of CFU/ml in which samples was vortexed does represent the true cell count.

(ii) Temperature. *Roseobacter* strain 27-4 grew well in MB at 15, 20, 25, and 30°C under static conditions with generation times of 3, 1.6, 1.4, and 1.3 h, respectively. Growth was slower at 10°C (generation time, 9.4 h) and did not occur at 5°C. *Roseobacter* cell numbers decreased rapidly at 37°C. The maximum cell density of 10^8 to 10^9 CFU/ml was reached at 15, 20, 25 and 30°C. Antibacterial activity was detected above cell concentrations of 3 to 5×10^8 CFU/ml. Activity in broth incubated at 20 and 25°C was detected up to 5 days in stationary phase when the experiment ceased. Activity levels of 64 to 128 arbitrary units were detected in cultures incubated at 15 to 25°C. At 30°C, however, the activity level was much lower, between 1 and 2 arbitrary units, despite cell densities equal to those at 15 to 25°C. No antibacterial activity was detected for cells incubated at 5, 10, and 37°C. Brown pigment was seen in all cultures when antibacterial activity was detected.

(iii) Salts. The pigment and antibacterial compound production of *Roseobacter* grown in MB did not appear to be a constant, reproducible feature. MB contains insoluble compounds (salts), and we hypothesized that these were important for pigmentation. If the MB media were sterile filtered, which removed the insoluble salts, significantly less pigment was formed than with unfiltrated MB. Low levels of antibacterial activity (2 to 8 arbitrary units) and no pigmentation were seen when *Roseobacter* grow to high cell densities (10^8 CFU/ml) in 2 and 3.3% IO.

(iv) AHL production. In the aerated cultures, AHL was detected by *A. tumefaciens* NT1(pZLR4) after 25 and 31 h at a cell density of 8×10^9 CFU/ml, but no AHL was detected thereafter. In static cultures AHL was detected by *A. tumefaciens* NT1(pZLR4) after 48 h of growth at a cell density of 9×10^8 CFU/g and remained at the same level for several days. No AHL induction of violacin was seen with *C. violaceum* CV026. The decrease in AHL concentration in the aerated cultures could be due to higher pH (pH 8.4) in the aerated culture compared to pH 6.9 in the static culture. However, AHLs also disappeared from these cultures when MB was buffered to 7.0. LC-ESI$^+$-HR-MS detected 3-hydroxy-decanoyl homoserine

TABLE 2. Antibacterial activity of supernatant from *Roseobacter* strain 27-4 after incubation at different temperatures

Temp (°C)	Zone (mm) in well diffusion assay[a] at:									
	15 min	Day								
		1	2	3	8	30	50	90	150	210
−80	ND	>22[b]	28	27	25	27	24	23	21	23
−20	ND	26	28	27	26	25	19	16	18	16
5	ND	25	25	26	22	ND	ND	ND	ND	ND
10	ND	21	21	23	19	ND	ND	ND	ND	ND
20	ND	22	20	19	10	0	ND	ND	ND	ND
25	ND	20	19	17	0	0	ND	ND	ND	ND
30	ND	19	16	9	0	0	ND	ND	ND	ND
37	ND	12	0	0	0	0	ND	ND	ND	ND
100	14	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Zones were measured in the well diffusion assay. ND, not determined.
[b] A freshly prepared supernatant results in 20- to 26-mm zones.

lactone with the same pattern of $[M+H]^+$, $[M+Na]^+$, $[M+H-H_2O]^+$ (low in source fragmentation) and protonated acetonitrile adduct of the aminooxohydrofuran moiety (*m/z* 143) (high in source fragmentation) as the reference standard, also at the correct retention time. The calculated accurate masses were all within the instrumental precision of approximately ±6 mDa.

Physical and chemical characteristics of antibacterial compound. The antibacterial compound produced by *Roseobacter*

strain 27-4 was not inactivated by exposure to different pH values (1, 3, 5, 7, and 9) for 1 to 1.5 h. In contrast, temperature influenced the antibacterial activity, which declined with increasing temperature (Table 2). For example, no activity was detected after 1 day incubation at 37°C, whereas activity was stable at 5°C for up to 8 days. The compound did not precipitate at sodium chloride concentrations of 5, 10, 15, or 30% (wt/vol).

The compound was extracted from a *Roseobacter* 27-4 culture grown under static conditions using acidified ethyl acetate extraction. LC-UV showed that the largest peak (Fig. 4) in the chromatogram had a UV spectrum that could be superimposed on the spectrum of tropodithietic acid published in Liang (23), with all four major absorptions matching, along with a valley and four shoulders. However, the UV data also matched the UV data of the related thiotropocin (37). LC-ESI$^+$-HR-MS showed that the accurate mass of the protonated molecular ion was 212.9605 Da. This was 6.4 mDa higher than 212.9669 Da, which is the theoretical mass both protonated thiotropocin and tropodithietic acid (same elementary composition), and within the instrumental precision. The compound also showed loss of CO_2, which is common for carboxylic acid and also observed by Liang (23). However, loss of CO_2 also appeared likely from thiotropocin and cannot be used to differentiate the two analogues. The isotope ratio between the $[M+H+2]^+$ and $[M+H+1]^+$ was 0.95, which matched the
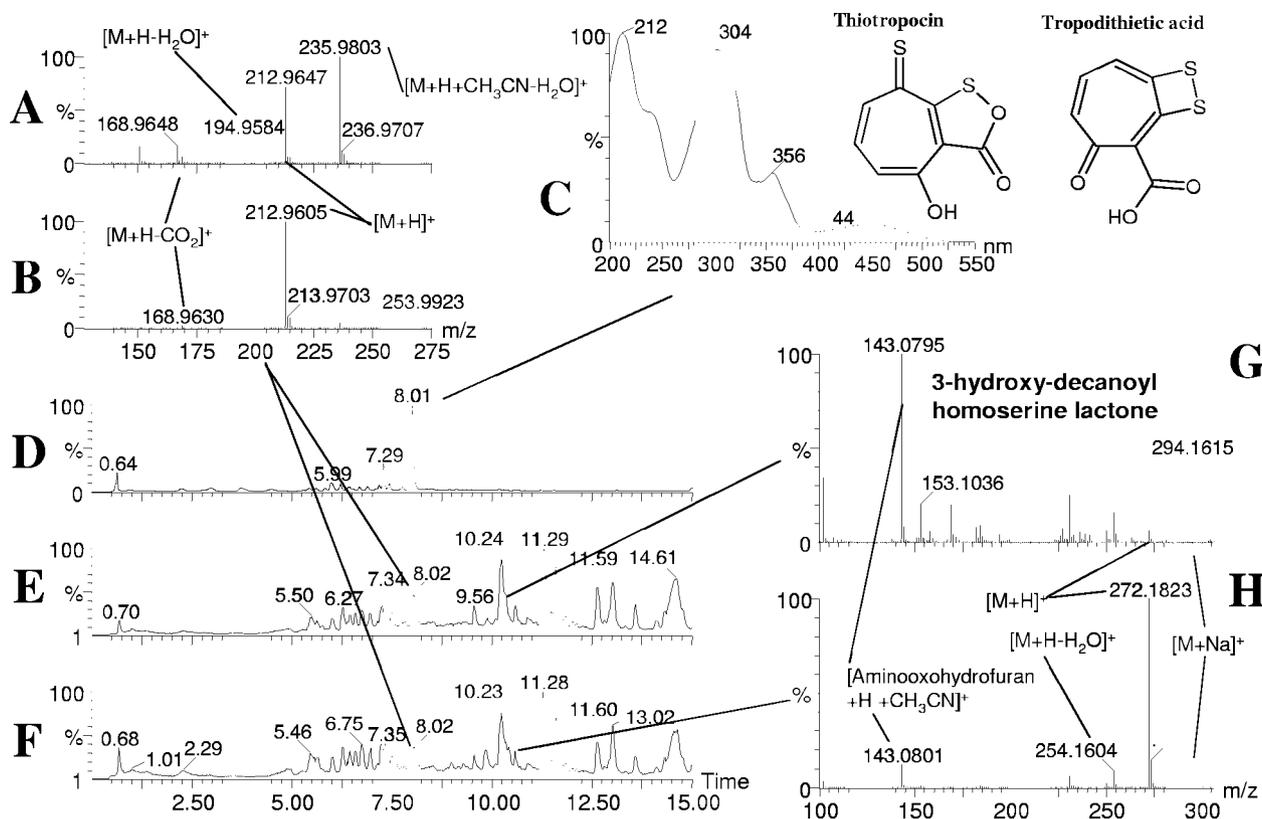

FIG. 4. Identification of tropodithietic acid and 3-hydroxy-decanoyl homoserine lactone in extract from *Roseobacter* strain 27-4 grown in MB. The ESI$^+$ mass spectra at high (A) and low (B) in-source fragmentation of tropodithietic acid/thiotropocin, along with the UV spectrum (C) and structures to the right. (D) UV chromatogram (200 to 700 nm). (E and F) ESI$^+$ total ion chromatograms at high and low in-source fragmentations, respectively. (G and H) ESI$^+$ spectra of 3-hydroxy-decanoyl homoserine lactone at high and low in-source fragmentations, respectively.
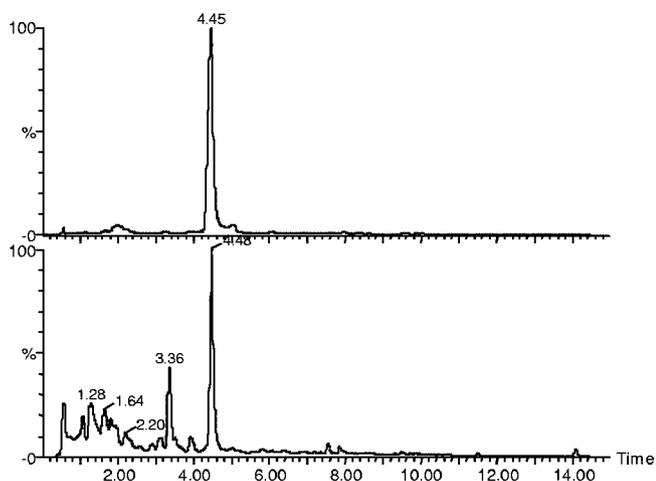
FIG. 5. UV chromatograms of before (lower) and after clean up on a Oasis MAX column of ethyl acetate extract of *Roseobacter* strain 27-4 grown in MB.

theoretical value of a disulfur compound of 0.96 (1.25 if containing three sulfurs and 0.55 if containing one). The presence of an acidic group was further validated as the compound eluted in the first acidic fraction (fraction 3) from the Oasis MAX column. In the crude extract the sulfur compound was the base peak in LC-UV chromatograms accounting for ca. 15% of the total peak area. After the singe Oasis Max clean-up step, it accounted for ca. 90% of the total peak area (Fig. 5). The sulfur compound could be detected with both ESI^+ and ESI^-, although compared to other microbial metabolites (28) it had quite poor response in both negative modes.

The brown pigment was retained on the Oasis MAX column even after the fraction 4, and we speculate that it could be a polyphenolic polymer. Since almost all bioactivity was found in fraction 3 and accounted for the same activity as the crude extract, it is clear that the pigment is not the active part and thus is only an indicator for activity. Also, it had a UV maximum of 398 nm where tropodithietic acid absorbs at 304 and 356 nm (Fig. 4).

DISCUSSION

Roseobacter species can produce antibacterial compounds (3) and are inhibitory to fish pathogenic bacteria (18, 32). Also, the live bacterial culture may reduce mortality of fish and fish larvae *Vibrio* species (18, 32). We show here that the antibacterial effect may be lethal since cells of *V. anguillarum* and *V. splendidus* were killed when exposed to supernatants of *Roseobacter*, and growth was strongly inhibited when exposed to supernatant of *Roseobacter* in MB media. However, the conditions required to induce the antibacterial activity are not known, and we describe here culture conditions required for *Roseobacter* strain 27-4 to be inhibitory against fish pathogenic bacteria.

The antibacterial compound produced by *Roseobacter* 27-4 is an extracellular compound as sterile filtered supernatants inhibited the fish pathogenic bacteria. Brinkhoff et al. (3) found that the antibacterial compound produced by *Roseobacter* T5 was tropodithietic acid and, in our study, LC-HR-MS con-

firmed the presence of thiotropocin or its precursor tropodithietic acid as the active compound of *Roseobacter* strain 27-4. The two *Roseobacter* strains are phylogenetically similar but not identical and have been isolated from quite different environments. Strain T5 has been isolated from an intertidal mud flat in the German Wadden Sea (3), whereas strain 27-4 has been isolated from a Spanish turbot farm just off the Galicean cost (18). Brinkhoff et al. (3) indicated that the activity seemed to be correlated to a brownish pigment since nonpigmented (mutant) colonies were devoid of antibacterial activity. Our *Roseobacter* strain 27-4 also produced a brownish diffusible compound, and antibacterial activity was only seen in pigmented cultures. However, the brownish pigment was not the primary bioactive compound since the full bioactivity of the extract was found in fractions and the pigment was not eluted from the column.

The sulfur containing compound thiotropocin or tropodithietic acid produced by *Roseobacter* strain 27-4 constituted as much as 15% of metabolite production, indicating that sulfur metabolism is a major secondary metabolic pathway. *Roseobacter* and other α-*Proteobacteria* play an important role in the sulfur cycle in the oceanic environment (27) where they degrade dimethyl sulfoniopropionate (DMSP) to dimethyl sulfide (DMS). DMSP is produced in high concentrations by phytoplankton, and the degradation product, DMS, is emitted to the atmosphere. Thus, *Roseobacter* species are crucial players in the global sulfur cycle. The substrate for formation of the antibacterial compound is not known but, assuming that DMSP can be used as substrate, it is likely to be produced when *Roseobacter* colonizes marine dinoflagellates and may be important for the dominance of the organism in this niche (1, 14).

Thiotropocin or tropodithietic acid was stable at different pH, whereas temperature influenced the activity, which declined rapidly with increasing temperature. Therefore, growth conditions ensuring a constant production of the antibacterial compound are needed if *Roseobacter* 27-4 is to be used as a probiotic bacterium in turbot rearing farms. *Roseobacter* strain 27-4 grew in a rather narrow temperature interval and did not survive 37°C. This narrow temperature window of growth corresponds to the findings by Selje et al. (33), who did not detect organisms from the *Roseobacter* clade in tropical and subtropical waters. In contrast, the *Roseobacter* and SAR11 clade constituted between 16 and 26% of rRNA gene clones retrieved from marine bacterioplankton in temperate to polar regions.

The antibacterial activity of *Roseobacter* strain 27-4 was only produced under specific conditions and only when grown in MB. The production of antibacterial substances by another *Roseobacter* strain BS107 was only seen if the culture was supplemented with supernatant of *V. anguillarum* (32). The antibacterial compound from *Roseobacter* BS107 was heat stable, indicating that the compound was different from tropodithietic acid or thiotropocin. The production of thiotropocin or tropodithietic acid in *Roseobacter* 27-4 was very sensitive to aeration since the antibacterial compound was only found under static conditions. *Roseobacter* BS107 was grown with agitation, which could have influenced the production of antibacterial compounds. In addition, aeration also caused a dramatic chance in the morphology of *Roseobacter* 27-4.

Static growth conditions caused *Roseobacter* strain 27-4 to grow in multicellular star-shaped mode, a similar behavior has

been described for a marine *Rugeria* strain (21) and marine *Agrobacterium* (30) now transferred to the *Roseobacter* clade (38). The purpose of this behavior, as well as the factors influencing this phenotype, is not known. However, stars were observed under the same conditions where antibacterial activity was detected, and it seems that the star shape is important for the organism to aggregate into a thick biofilm. *Roseobacter* species are excellent biofilm formers and are the first to colonize surfaces in open waters (9). *Roseobacter* strain 27-4 is probably also an excellent biofilm-forming bacteria since it has primarily been isolated from tank walls (18) where the same subtype has been persisting over 1 year (19). The attaching and biofilm-forming capacity may be important for survival in the marine environment and may be characteristics that allow the bacterium to colonize algae (1).

The antibacterial compound was only detected at high cell densities, and this could indicate that the production was controlled in a quorum-dependent manner. Indeed, quorum sensing has been shown to control the production of antibacterial compounds in several bacteria (2, 36, 42). The probiotic bacteria *P. aureofaciens*, which is used to protect wheat against filamentous fungi, produce the quorum-sensing signal molecule *N*-hexanoyl-homoserine lactone, and this molecule is present and responsible for expression of three antibiotics in situ on plant roots (41). *Roseobacter* strains isolated from marine snow or dinoflagellates produce compounds that induce AHL reporter systems (4, 15, 22); however, the exact nature of the compounds have not been elucidated. In the present study, we detected *N*-(3-hydroxydecanoyl)-L-homoserine lactone from *Roseobacter* strain 27-4. This compound has been previously tentatively identified by its ESI-MS/MS spectrum in the strain *P. fluorescens* 2-79 (34) and is also produced by *Burkholderia pseudomallei* (39). It is, however, not known which phenotypes are regulated by the quorum-sensing systems. The production of AHL may also affect the probiotic ability of *Roseobacter* to prevent infections of fish pathogenic since many fish pathogenic bacteria have also been shown to produce AHL signal molecules (6), and cross talk between different bacteria, which produces signal molecules is expected. Further studies are needed to determine whether the AHL compound is involved in the regulation of the antibacterial activity of *Roseobacter* strain 27-4. These could be determined by comparing the wild-type strain with *luxI/R* mutants of *Roseobacter* 27-4.

One may also hypothesize that the multicellular star-shaped growth behavior of *Roseobacter* strain 27-4 is influenced by quorum sensing. The cell morphology of the yeast *Candida albicans* is affected by AHL compounds (20), and the aggregation into mature biofilms is dependent on quorum sensing in several bacteria such as *Aeromonas hydrophila* (24) and *Vibrio anguillarum* (8). However, stars were formed during growth at low and high cell densities, which was not expected if the star forming was regulated by a quorum sensing system.

If *Roseobacter* 27-7 is to be used as a probiotic bacteria in the aquaculture sector, a further understanding of the effects of the antibacterial compound are needed. Especially, it must be determined if the multicellular behavior and the aggregation and biofilm formation are essential for the antibacterial activity. Therefore, the interaction between *Roseobacter* 27-4 and fish pathogenic bacteria have to be studied under conditions closer to the in vivo situation. Since the *Roseobacter* both in its

natural dinoflagellate niche in the oceans and during production of turbot is mostly found attached to a surface (1, 19, 26), studies of interactions between *Roseobacter* and other bacteria, including fish pathogenic bacteria, must be carried out in biofilm environments. This would require setup of appropriate model systems and development of methods, allowing specific quantification of *Roseobacter* and fish pathogenic bacteria during attachment to surfaces and on dinoflagellates.

In conclusion, *Roseobacter* 27-4 has the ability to be used as a probiotic bacterium in fish farms since it inhibits and kills fish pathogens bacteria. Production of the antibacterial compound was growth specific and was only produced in MB under static conditions. A constant production of thiotropocin or tropodithietic acid must be assured since the compound is unstable, and *Roseobacter* 27-4 must therefore be established under specific conditions when it is to be used as a probiotic culture. Further studies on the physiology of *Roseobacter* will also have greater importance due to its role in the oceanic environment.

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