Culture Conditions of *Roseobacter* Strain 27-4 Affect Its Attachment and Biofilm Formation as Quantified by Real-Time PCR

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The fish probiotic bacterium *Roseobacter* strain 27-4 grows only as rosettes and produces its antibacterial compound under static growth conditions. It forms three-dimensional biofilms when precultured under static conditions. We quantified attachment of *Roseobacter* strain 27-4 using a direct real-time PCR method and demonstrated that the bacteria attached more efficiently to surfaces during static growth than under aerated conditions.

Bacteria belonging to the marine *Roseobacter* clade may produce antibacterial compounds (4, 5, 6) and have been suggested for use as a probiotic treatment in aquaculture (12, 19, 23, 24). *Roseobacter* strains were isolated from a turbot rearing facility, and very similar DNA types were repeatedly isolated from tank walls over a 1-year period (13). One of these strains, *Roseobacter* strain 27-4, inhibits fish pathogenic bacteria by a sulfur-containing antibacterial compound (5, 12) and improves the survival of turbot larvae infected with *Vibrio anguillarum* (19). The antibacterial compound is produced only under static growth conditions, where the bacterium forms a thick biofilm at the air-liquid interface. The biofilm produced by *Roseobacter* strain 27-4 appears to be correlated to a multicellular growth pattern in which 9 to 10 cells grow in a rosette shape. The rosette is unstable, and shaking will break up the structure; during growth under aerated conditions, the organism grows as single cells and no inhibitory activity is detected (5).

It is not known if the probiotic *Roseobacter* strain 27-4 retains its antibacterial properties when it attaches to inert surfaces; however, marine bacteria such as *Roseobacter gallaeciensis* and *Pseudoalteromonas tunicata* can prevent other bacteria from forming a biofilm (20). We hypothesize that interaction between *Roseobacter* and fish pathogenic bacteria may take place on surfaces in biofilms and that it potentially can exert its effect as a probiotic bacterium by colonizing the turbot larva rearing facilities. Here we describe the development of a real-time PCR method that allows quantification of *Roseobacter* strain 27-4 on solid surfaces. We also describe how culture conditions may influence biofilm formation of *Roseobacter* strain 27-4.

Effect of preculture growth conditions on pigmentation. In this study, as in previous studies (5), *Roseobacter* strain 27-4 grown in marine broth (MB) (Difco 279110) under static conditions grew as rosettes consisting of 9 to 10 cells, produced a

brown pigment, and formed a biofilm at the air-liquid interface. However, if the statically grown preculture was vigorously shaken before inoculation or if an aerated preculture was used, almost no pigment and biofilm formation were produced (data not shown). Furthermore, when an aerated preculture was used, almost no rosette-forming cell clusters were observed even after several days of static growth.

***Roseobacter* strain 27-4 biofilm formation on plastic surfaces.** Statically grown overnight cultures of *Roseobacter* strain 27-4 grown in MB were diluted to an optical density at 600 nm (OD₆₀₀) of 0.065 in MB, and 100- μ l culture aliquots were dispensed into the wells of a 96-well polystyrene microtiter plate (Nunc 163320) and incubated at 25°C. Biofilm formation was measured using staining with 1% (wt/vol) crystal violet (17, 18). A statically grown preculture of *Roseobacter* strain 27-4 adhered readily to the surface, and the crystal violet OD increased to an OD₅₉₀ of 0.59 after 24 h (Fig. 1).

***Roseobacter* strain 27-4 biofilm formation as assessed by confocal laser scanning microscopy.** Biofilms were grown at room temperature in three-channel flow chambers (8). Each channel was supplied with a flow of 0.85 ml/h of MB medium diluted 10-fold. Precultures of *Roseobacter* strain 27-4 were inoculated in MB and grown under static or aerated (200 rpm) conditions for 2 days at 25°C. The cultures were diluted 10-fold in 0.9% NaCl, and 250 μ l was inoculated into each flow channel using a small syringe. The flow was resumed after 1 h with a mean flow velocity in the flow cells of 0.057 mm/s using a Watson Marlow 205S peristaltic pump (Wilmington, Mass.). *Roseobacter* strain 27-4 was stained at different time points using a fluorescent stain, SYTO9 (Molecular Probes, Oregon) and observed using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) (8).

The rosette structures from stagnant precultures immediately attached to the glass surface, and the number of attached rosettes increased over 5 days, resulting in a multilayered biofilm (Fig. 2a). From the aerated preculture, only single cells or small clusters of cells were observed (Fig. 2b). The increased biofilm capacity of the stagnant culture was observed several times; however, occasionally the multilayer biofilm did not form. The multilayered structure was never seen when the inoculum was from an aerated culture.

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FIG. 1. Biofilm formation of *Roseobacter* strain 27-4 on polystyrene surfaces. *Roseobacter* strain 27-4 was grown in MB at 20°C, and the amount of biofilm was determined by a crystal violet assay.

Several bacteria belonging to the *Roseobacter* clade attach to surfaces (1, 9), and the rosette morphology has been described in other related bacteria (15, 22); however, this morphology has not been linked to biofilm formation before. When *Roseobacter* strain 27-4 grows in the rosette fashion, no flagella are seen, whereas planktonic bacteria are motile (5). The effective attachment of the rosette may be caused by the slime layer, which was observed by scanning electron microscopy for *Roseobacter* strain 27-4 grown under static conditions (5).

Primer design, DNA extraction, and real-time PCR procedures. To be able to quantify attachment and biofilm formation, we set up a new system whereby the attached cells were quantified by real-time PCR. Primers that discriminated *Roseobacter* strain 27-4 from several co-occurring fish pathogenic bacteria were designed within the 16S rRNA gene (GenBank accession number AJ536669) by an alignment with 16S rRNA genes from the following fish pathogenic bacteria: *Aeromonas salmonicida* NCIMB 1102 (GenBank accession number X60405), *Aeromonas hydrophila* ATCC 7966 (X60404), *V. anguillarum* NB10 (M28386), *Vibrio damsela* ATCC 33539 (AY191126), and *Vibrio splendidus* strain VS6 (AJ132988). The forward primer, 5'-ACGTGCCCTTCTCTAAGGAATA-3', and the reverse primer, 5'-CCGATCCTTCTCCGATAAATCT-3', were synthesized by DNA Technology A/S, Denmark, and produced an 89-bp product. One milliliter of an outgrown culture was boiled for 10 min (Techen DB-2A Block Heater, Cambridge, England). The samples were centrifuged at $15,000 \times g$ for 3 min, and DNA was extracted with a Macherey-Nagel NucleoSpin Tissue kit (M740952) according to the manufacturer's protocol. Samples were stored at -20°C, thawed, and diluted 10-fold in sterile MilliQ water before PCR amplification.

All PCR amplifications were performed from 10 μ l of diluted purified genomic DNA using Brilliant SYBR Green QPCR Master Mix (catalog no. 600548; Stratagene, La Jolla, Calif.), with ROX (6-carboxy-X-rhodamine) as a reference dye, in a 25- μ l reaction volume containing 0.54 pmol of each primer. The PCR amplification (10 min at 95°C and then 40 cycles of three steps consisting of 30 s at 95°C, 60 s at 62°C, and 30 s at 72°C) was performed with an MX3000P (Stratagene, La

FIG. 2. Confocal micrographs showing *Roseobacter* strain 27-4 biofilm development after 5 days when the inoculum was from a stagnant (A) or an aerated (B) preculture. Images are three-dimensional projections. Bar (lower left corner), 10 μ m.

Jolla, Calif.) instrument. All samples were processed for melting curve analysis. The primers detected *Roseobacter* strain 27-4 with a cycle threshold (C_T) value of 14, and none of the fish pathogenic bacteria were detected (C_T of >40). Five other bacteria from the *Roseobacter* clade also gave PCR positive results, albeit delayed, with the designed primers.

Bacteria may interact during attachment and in biofilms (3, 7, 11), and antagonistic interactions during attachment have been observed with bacteria belonging to the *Roseobacter* clade (20). The primers designed for *Roseobacter* strain 27-4 did not

FIG. 3. Standard curve representing the correlation between CFU/ml of *Roseobacter* strain 27-4 and the C_T value detected with the real-time PCR method.

detect any of the pathogenic bacteria, and the real-time PCR setup can therefore be used in this model system to quantify both *Roseobacter* strain 27-4 and fish pathogenic bacteria attached to a surface. *V. anguillarum* and *V. splendidus* are killed by *Roseobacter* supernatant (5), and the DNA of dead bacteria must therefore be removed or bound to ethidium monoazide (21) before the procedure is used in systems where there is interaction between bacteria.

Standard curve relating cell numbers to real-time PCR C_T values for *Roseobacter* strain 27-4. *Roseobacter* strain 27-4 was grown in 150 ml of MB for 4 days under aerated conditions and serially diluted. DNA was extracted from duplicate samples from each of the 10-fold dilutions, and C_T values were determined in the real-time PCR procedure. C_T values and CFU/ml (determined on marine agar) for each dilution step were compared by linear regression and resulted in a linear correlation coefficient (R^2) of 0.991 (Fig. 3).

Optimization of DNA extraction from attached *Roseobacter* strain 27-4 and testing by real-time PCR. Quantification of attached bacteria by real-time PCR requires that the bacteria are lysed and release their DNA. *Roseobacter* strain 27-4 was allowed to attach to stainless steel coupons for 4 days at 25°C under static conditions (2, 16). Coupons were removed in duplicates, and nonattached and poorly attached bacteria were removed by rinsing the coupons (2). The coupons were boiled

in Eppendorf tubes for 10, 30, or 60 min in 1.4 ml of water or sodium dodecyl sulfate (SDS) solution (catalog no. 17-1313-01; Pharmacia Biotech, Sweden) at 0, 0.1, 1, or 2%. The coupons were removed, and DNA was harvested ($15,000 \times g$ for 3 min). The supernatant was removed, and the samples were stored at -20°C before DNA extraction and real-time PCR. Since the lowest C_T value was obtained by a 10-min treatment without SDS (Table 1), this procedure was used in the growth experiments. The removal of bacteria was confirmed by DAPI (4',6'-diamidino-2-phenylindole) staining of the coupons.

Quantification of growth and adhesion by *Roseobacter* strain 27-4 in a batch system. Stainless steel disks were submerged in 250 ml of MB and left stagnant or aerated by a magnet stirring at 500 rpm. *Roseobacter* strain 27-4 was inoculated at 5×10^2 CFU/ml. Samples of liquid culture and stainless steel coupons were removed at regular intervals, and cell densities in culture and on the coupons were determined. Cell densities in broth were determined by plate counting (on marine agar for 4 days at 25°C) and by real-time PCR (in duplicates). Cell density increased to 5×10^8 CFU/ml in the static culture (Fig. 4A) and to 5×10^9 CFU/ml in the aerated culture (Fig. 4B). Real-time PCR estimates of cell counts were similar to plate counts both in the aerated and the statically grown cultures (Fig. 4).

Cells were lysed on coupons by 10 min of boiling in distilled water, with subsequent quantification by real-time PCR. The number of attached bacteria was also estimated by DAPI staining (2 μg per ml for 5 min; Sigma D-9542) (2). Four pictures were taken of each coupon, and the cell numbers were estimated.

Roseobacter strain 27-4 attached almost immediately to stainless steel coupons, and between 35 and 100 CFU/cm² were detected using the real-time PCR procedure (Fig. 4). The highest absolute number of attaching bacteria was seen in the static culture, which reached 1×10^7 CFU/cm² as opposed to 2×10^6 CFU/cm² in the aerated culture ($P < 0.05$), even though the cell density in the aerated culture was 10-fold higher. The standard deviations of the real-time PCR C_T values were very low in both the static and aerated cultures. DAPI counts (above 10^5 CFU/cm²) were in good agreement with the cell densities estimated by real-time PCR in both the aerated and static cultures (Fig. 4).

During growth of the static culture, a brown pigment formed after 32 h, whereas no pigment was seen in the aerated culture. The growth of cells in rosette structures was seen in the liquid phase of the static culture; however, DAPI staining of the stain-

TABLE 1. C_T values from cells attached to stainless steel coupons and lysed under different boiling conditions^a

Boiling time (min)	Expt no. ^b	C_T value after boiling coupons in different concentrations of SDS			
		0%	0.1%	1%	2%
10	1	12.94 $\pm$ 0.16	16.17 $\pm$ 0.03	17.07 $\pm$ 1.28	16.20 $\pm$ 0.37
	2	14.95 $\pm$ 0.33	15.37 $\pm$ 0.44	17.24 $\pm$ 1.20	
30	1	17.23 $\pm$ 1.60	20.00 $\pm$ 0.74	19.31 $\pm$ 1.54	20.47 $\pm$ 0.91
	2	15.95 $\pm$ 1.70	19.41 $\pm$ 2.71	19.65 $\pm$ 0.29	
60	1	17.80 $\pm$ 4.68	21.39 $\pm$ 0.26	19.17 $\pm$ 1.16	17.02 $\pm$ 1.45
	2	18.75 $\pm$ 0.67	23.94 $\pm$ 0.18	23.47 $\pm$ 0.48	

^a The C_T value in each experiment is the mean of results from two coupons from which two real-time PCR cycles have been run.

^b Individual growth experiments were run in duplicates.

FIG. 4. Quantification of *Roseobacter* strain 27-4 in liquid culture (MB) and attached to stainless steel coupons under static conditions (A) and under aerated conditions (B). □, CFU/ml determined by plate counts; ■, CFU/ml determined by real-time PCR (in duplicates); ○, CFU/cm² attached bacteria determined by DAPI staining; ●, CFU/cm² attached bacteria determined by real-time PCR (in duplicates).

less steel plates revealed no attaching rosettes of *Roseobacter* strain 27-4, probably due to the rinsing of the plates.

Real-time PCR has been used for quantification of bacteria in, e.g., dental plaque (25) or adherent gastrointestinal mucosa (14), and a recent study quantified *Listeria monocytogenes* directly on an inert surface by real-time PCR (10). The limit of detection was 6×10^2 cells per cm²; however, due to a very large standard deviation at low cell counts, the method did not allow quantification below approximately 10^4 cells per cm², which is 10- to 100-fold less sensitive than our method.

Guilbaud et al. (10) prepared a standard curve by comparing C_T values of DNA extractions from adherent cells with plate counts from cells removed by sonication. This standard curve was different from a C_T -CFU/ml standard curve based on dilutions of *L. monocytogenes* DNA (10). Also, we found that our C_T -CFU/ml standard curve based on dilution of DNA from a fully grown *Roseobacter* strain 27-4 culture was different from the standard curve based on extracting DNA from each 10-fold dilution of the bacteria. Validation of the real-time PCR method by monitoring *Roseobacter* strain 27-4 during growth in liquid medium showed that only the standard curve based on

DNA preparations from a dilution of cells resulted in cell counts similar to the one based on plate counts (data not shown). Hence, such a standard curve should be used when quantifying bacteria.

Conclusions. In the present study, we have demonstrated that *Roseobacter* strain 27-4 attaches to and forms a biofilm on inert surfaces. The amount of biofilm and the three-dimensional structure of the biofilm were highly influenced by the culture conditions of the organism and were linked to the ability of the bacterium to grow in a rosette-like fashion. The production of an antibacterial compound occurs when rosettes are formed (5), and we therefore believe that this phenotype is important for the bacterium's use as a probiotic organism. If *Roseobacter* strain 27-4 is to be effective in reducing vibriosis in turbot rearing facilities, one must ensure that the preculture contains the rosette shape of the bacteria. We developed a method that allowed direct quantification of bacteria on a surface. If species-specific primers are developed, such methods are not only useful in microbial ecology but also required to evaluate, for instance, novel antifouling surfaces or novel cleaning and disinfection methods for removing bacteria attached or in a biofilm.

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