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Survival and Activity of *Pseudomonas* sp. Strain B13(FR1) in a Marine Microcosm Determined by Quantitative PCR and an rRNA-Targeting Probe and Its Effect on the Indigenous Bacterioplankton

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Genetically engineered *Pseudomonas* sp. strain B13(FR1) was released into laboratory-scale marine ecosystem models (microcosms). Survival of the introduced population in the water column and the sediment was determined by plating on a selective medium and by quantitative competitive PCR. The activity of the released bacteria was determined by in situ hybridization of single cells with a specific rRNA-targeting oligonucleotide probe. Two microcosms were inoculated with 10^6 cells ml^{-1}, while an uninoculated microcosm served as a control. The number of *Pseudomonas* sp. strain B13(FR1) cells decreased rapidly to ca. 10^2 cells ml^{-1} within 2 days after the release, which is indicative of grazing by protozoa. Three days after the introduction into seawater, cells were unculturable, but PCR continued to detect cells in low numbers. Immediately after the release, the ribosomal content of *Pseudomonas* sp. strain B13(FR1) corresponded to a generation time of 2 h. The growth rate decreased to less than 0.04 h^{-1} in 5 days and remained low, probably because of carbon limitation of the cells. Specific amendment of the microcosms with 10 mM 4-chlorobenzoate resulted in a rapid increase of the growth rate and an exponentially increasing number of cells detected by PCR, but not in resuscitation of the cells to a culturable state. The release of *Pseudomonas* sp. strain B13(FR1) into the microcosms seemed to affect only the indigenous bacterioplankton community transiently. Effects on the community were also apparent from the handling of water during filling of the microcosms and the amendment with 4-chlorobenzoate.

The survival and activity of nonindigenous bacteria released into the environment are governed by physical, chemical, and biological parameters. To colonize a new environment, a released bacterial population must be able to withstand the stress induced by restricted nutrient availability (36), predation (19), and suboptimal physical conditions (4, 34, 47). The microbial response to environmental stress varies among species, from rapid elimination of the population to persistence over prolonged periods (33).

The effect of a single environmental parameter on the fate of the released microorganisms can be studied in simple experimental systems. However, the combined effects of all parameters can be assessed only in complex ecological model systems, or microcosms (13, 15, 32). A further requisite for determining microbial fate in the environment is use of sensitive methods for quantitating population dynamics. Selective plating is the most widely used method for detecting and quantitating microorganisms in the environment. However, the plating method fails to detect bacteria that become nonculturable in response to environmental stress (45). The presence of nonculturable cells in the environment has been confirmed by detection of the genotype by PCR (5, 7). However, PCR has not been used quantitatively, because of variations of the amplification process between individual reactions. By using an internal standard, which is coamplified with the target fragment in a competitive PCR (cPCR), this reaction-to-reaction variation can be accounted for, enabling quantitation from a standard curve (31). Oligonucleotide probes targeting rRNA are attractive for in situ identification of bacteria, since they can be designed to be specific for individual strains (11, 50) and since rRNA is naturally amplified in growing cells (8). A linear relationship between the RNA content and the growth rate has been demonstrated for different bacteria (29, 48). The number of ribosomes (i.e., the metabolic activity) can be estimated by probing with an rRNA-targeting fluorescent probe (10, 41) and by quantitating the fluorescence intensity from single cells by use of a charged coupled device (CCD) camera.

In the present study, we studied the survival and activity of a bacterial strain released into marine microcosms using classical and molecular methods. The model organism used was *Pseudomonas* sp. strain B13(FR1), a genetically modified strain capable to grow on various chloroaromatics. The fate of the strain in the marine environment was studied, since this environment ultimately may receive a load of *Pseudomonas* sp. strain B13(FR1) if the strain is released for bioremediation purposes. Long-term survival and viability of the parental strain (*Pseudomonas* sp. strain B13) and its derivatives have been documented for freshwater sediments (40), soil (9), and aquifer material (51). Microcosms were amended with 4-chlorobenzoate (4CB) to study the effects of a selective enrichment on the released strain. Additionally, we studied the effects of the release on the indigenous bacterioplankton by DNA melting profiles. This approach of comparing the genetic compositions of community DNA, on the basis of the base composition of the DNA, was used successfully by Holben et al. (27) and by Harris (21) to characterize DNA extracted from soil.

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MATERIALS AND METHODS

Bacterial strain. *Pseudomonas* sp. strain B13(FR1) is a genetically modified strain of *Pseudomonas* sp. strain B13, which can grow on 4CB as the sole carbon source (44).

Microcosm experiment. The three microcosms used were a modified version of the microcosm described by Leser (32). Each microcosm consisted of a pelagic part and a sediment part. The pelagic part was contained in a 216-liter glass unit. Three units of acrylic tubes (diameter, 16 cm) contained intact sediment cores overlaid by 5 to 10 cm of water. Water was circulated continuously between the two compartments by peristaltic pumps. Light was supplied by 12 white fluorescent lamps (Philips TL30), which provided an intensity at the water surface of 340 μE m^{-2} s^{-1} of photosynthetically active radiation. The microcosms were maintained at 17°C.

The microcosms were filled with freshly collected water from the pier at Risø, Denmark in Roskilde Fjord as previously described (32). Water was collected at approximately 1 m below the surface. Sediment cores (ca. 20 cm deep) were collected from shallow depths. The experiment was done in June 1993. The water temperature on the sampling day was 17°C.

Pseudomonas sp. strain B13(FR1) was grown in LB medium (6) to early stationary phase, harvested by centrifugation, washed twice in M9 buffer (46), and finally resuspended in M9 buffer. The number of cells in the inoculum was determined by acridine orange (AO) direct counts. Two microcosms (M1 and M2) were inoculated by addition of the cell suspension to yield approximately 10^6 cells ml^{-1}. The third, uninoculated microcosm (M3) served as a control. The microcosms were inoculated with cells 2 days after the initial filling of the microcosms.

At 13 days after the release of *Pseudomonas* sp. strain B13(FR1), all microcosms were amended with 4CB to a concentration of 10 mM.

***Pseudomonas* sp. strain B13(FR1) CFU.** The number of culturable *Pseudomonas* sp. strain B13(FR1) cells was determined by plating diluted samples in triplicate on M9 medium supplemented with mineral salts (53) and kanamycin (100 μg ml^{-1}), with 5 mM 4CB as the carbon source. Colonies were counted after 3 days at 30°C.

Sediment was sampled by using syringes with the tips cut off. A syringe was pushed into the sediment and its piston was retracted to yield a volume of 1.5 ml. From each microcosm, samples from each of the three sediment cores were pooled. This was done in duplicate. The pooled samples were vigorously mixed by vortexing. Subsamples (1 g) were diluted for plate counts. The sediment dry weight was determined (105°C overnight).

The total number of bacteria in the water column was determined by AO direct counts (25).

Quantitation of *Pseudomonas* sp. strain B13(FR1) by cPCR. (i) DNA extraction from water and sediment. DNA was extracted and purified from the water as previously described (31). From the pooled sediment samples (the same samples as those used for CFU counting), DNA was extracted by direct lysis of cells. Subsamples (100 mg [wet weight]) were transferred to 1.5-ml centrifuge tubes, and 500 μl of TENP (50 mM Tris, 20 mM EDTA [pH 8.0], 100 mM NaCl, 1% [wt/vol] polyvinylpolyporrolidone [Sigma Chemical Co., St. Louis, Mo.] buffer (39) was added. The samples were then subjected to five rapid freeze-thaw cycles, each consisting of freezing for 2 min in a dry ice-ethanol bath and heating for 2 min at 70°C during shaking at 1,400 rpm. After centrifugation at 2,800 $\times$ *g* for 10 min at 4°C, the pellets were washed by vortexing with 100 μl of TENP, and the suspensions were centrifuged as described above. The two supernatants were pooled and centrifuged at 8,000 $\times$ *g* for 15 min at 4°C. DNA extracts were purified using CTAB (hexadecyltrimethyl ammonium bromide) as previously described (31).

(ii) Quantitative PCR. *Pseudomonas* sp. strain B13(FR1) was quantitated by cPCR as described by Leser (31). Briefly, a 712-bp fragment (B13 fragment) specific for *Pseudomonas* sp. strain B13(FR1) (51) was coamplified with a 588-bp internal standard which has the same priming sequences as the B13 fragment. A two-temperature cycle was used. A 2-min initial denaturation period at 94°C was followed by 30 cycles (water samples) or 40 cycles (sediment samples), consisting of a 2-min denaturation at 94°C and 2-min primer annealing and extension at 72°C, with a final 7-min extension period at 72°C. The reaction conditions were as described previously (31). For amplification of sediment samples, 0.65 μg of T4 gene *32* protein (Boehringer, Mannheim, Germany) was added to the reaction mixtures. Following cPCR, the two fragments were separated by gel electrophoresis and photographed, and the fragments were quantitated from digitized images with the Cream program (20). When low numbers of *Pseudomonas* sp. strain B13(FR1) were present, increased detection sensitivity was obtained by hybridizing Southern blots with a 345-bp digoxigenin-labeled probe (31).

Standard curves for cPCR were made by the addition of 10-fold dilutions of a known amount (AO direct count) of *Pseudomonas* sp. strain B13(FR1) to 1-liter water samples or 100-mg sediment samples. DNA was extracted by the same procedure as that used for the microcosm samples and was amplified with a constant amount of internal standard. Separate standard curves were made for water and sediment by plotting the density of *Pseudomonas* sp. strain B13(FR1) on the abscissa and the ratio of amplified B13 fragment to internal standard products on the ordinate.

In situ hybridization. (i) Oligonucleotide probes. The oligonucleotide probes used in this study were as follows: EUB338 (17), 5′-GCTGCCTCCCGTAGG AGT-3; non-EUB338, (a negative control probe [10]), 5′-CGACGGAGGG CATCCTCA-3′; and Ps-B13, 5′-CCGATTCCCGAAGGCACTCTC-3′. Ps-B13 is a probe specific for *Pseudomonas* sp. strain B13(FR1) targeting nucleotides 1020 to 1039 in 16S rRNA (*Escherichia coli* numbering). The oligonucleotide probes were synthesized by the Center for Microbial Ecology, Denmark, by using a Pharmacia Automatic DNA Synthesizer. The specificities of the probes were checked against the Ribosomal Database Project database (30) and by whole-cell hybridizations to closely related pseudomonads.

(ii) Continuous culture. *Pseudomonas* sp. strain B13(FR1) was grown in continuous culture under carbon limitations, except for the growth rate of 1.25 h^{-1} obtained in a batch culture with LB medium. The chemostat was coated with 2% dichlorodimethylsilan (Fluka, Buchs, Switzerland) to prevent cells from attaching to the wall. M9 medium supplemented with 0.02% glucose (wt/vol) and mineral salts (53) was used. Carbon was controlled by adjusting the in-flow rate of fresh medium to the chemostat. Culture density was determined once per day by measuring the optical density at 450 nm. Culture purity was assessed by microscopy and plating on M9 medium agar plates. The temperature was 22°C. Each culture was maintained at steady state (constant optical density) for 7 to 10 generations before harvesting. The growth rate at steady state was calculated by standard chemostat equations (24).

(iii) Fixation and hybridization for epifluorescence microscopy. Cultured cells were fixed in 4% paraformaldehyde as previously described (2) and were stored at −20°C in storage buffer (50% ethanol, 10 mM Tris [pH 7.2], 0.1% Nonidet P-40) for less than a month. Cells were bound to gelatin-coated slides containing six wells (4) and were dried by sequential washes in 50, 80, and 100% ethanol (3 min each). Following the ethanol series, 10 μl of hybridization buffer (30 to 40% formamide, 0.9 M NaCl, 100 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate, [SDS]) containing 25 ng of probe was added to each well. Formamide (30%) was used for the EUB338 and non-EUB338 probes, and 40% formamide was used for the Ps-B13 probe. The cells were hybridized for 16 h in a moisture chamber (2) and were washed for 15 min at 37°C in 100 ml of hybridization buffer without probe. The cells were 4′,6-diamidino-2-phenylindole (DAPI) stained in 100 ml of washing buffer with 25 μg of DAPI ml^{-1} for 15 min at 37°C and were washed in 100 ml of washing buffer (0.9% NaCl, 100 mM Tris [pH 7.2]). Finally, the cells were rinsed with water and air dried.

Bacteria from 40-ml samples collected from the pelagic part of the microcosms were pelleted by centrifugation (8,000 $\times$ *g*, 20 min), fixed as described above, and finally resuspended in 1 ml of storage buffer. The hybridization and washing conditions were as described above.

(iv) AO staining. The cells were fixed, bound to gelatin-coated slides, and dehydrated in ethanol series as described above. A 40-μl volume of a filtered (0.22-μm-pore-size filter) AO-staining solution (22 μM AO, 5 mM EDTA, 0.15 M NaCl, 0.1 M phosphate-citrate buffer [pH 6.0]) was applied to each well. The slides were incubated in the dark for 5 min, washed for 30 s in 100 ml of H_2O double-distilled and air dried.

(v) Epifluorescence microscopy and image analysis. A Carl Zeiss Axioplan microscope equipped for epifluorescence and phase-contrast microscopy was used. The microscope was equipped with a 100-W mercury lamp and filter sets 487715, 487710, and 487701 (Zeiss, Oberkochen, Germany) for TRITC (tetramethyl rhodamine isothiocyanate), fluorescein isothiocyanate, and DAPI, respectively. Filter set XF21 (Omega Optical, Brattleboro, Vt.) was used for AO. A narrow-bandpass filter, Corion SS 590 (585 to 595 nm [L.O.T. Oriel, Darmstadt, Germany]), was used in combination with filter set 487715. The microscope was attached to a Peltier-cooled slow-scan CCD camera (CH250) with a KAF 1400 chip (Photometrics, Munich, Germany). Digital images were acquired as 12-bit images with the program Pmis version 1.5 (Photometrics). The integration time for the CCD camera was 500 ms for ordinary filter sets and 2 s when the narrow-bandpass filter was used. The images were subsequently exported as 12-bit files, which were analyzed with the NIH Image version 1.51 program running on a Macintosh Quadra 950. Quantitation of fluorescence intensity and measurement of cell area were done by manually circumscribing the edge of the cell with a pointing device.

Samples were mounted in Zeiss emission oil, except for samples hybridized with the fluorescein-labeled probe. These were mounted in Citifluor (University of Kent, Canterbury, England).

The hybridizations were counterstained with DAPI to facilitate focusing of the camera without bleeding the specific signal from the rRNA-targeting probe.

DNA-melting profiles. (i) Source of bacterioplankton. Samples (10 liters) of naturally occurring near-surface planktonic bacteria were collected in plastic carboys from the pier at Risø in Roskilde Fjord on 10 occasions during 1993. From the microcosms, 8-liter water samples were siphoned into plastic carboys. All samples were further processed within 3 h after sampling.

(ii) DNA extraction and purification. DNA was extracted by a modification of the method described by Fuhrman et al. (16), as described by Hendriksen (23). Briefly, bacteria from the water samples (8 or 10 liters) were collected after prefiltration, excluding eucaryotic cells, on 0.22-μm-pore-size membrane filters. The filters were frozen immediately and kept at −20°C until DNA extraction. The bacteria on the filters were lysed with hot 10% SDS after digestion with lysozyme (Sigma) and pronase E (Sigma). The DNA was then purified by ethanol precipitation and then by cesium chloride-ethidium bromide equilibrium density centrifugation and purification with CTAB (only samples from microcosms).

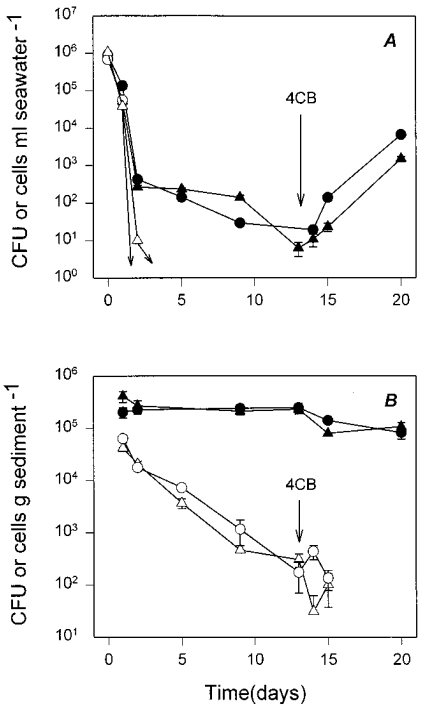

FIG. 1. Survival of *Pseudomonas* sp. strain B13(FR1) in the water column (A) and in the sediment (dry weight) (B) after release into microcosms. CFU were enumerated on selective medium (open symbols). Numbers of cells were determined by cPCR (closed symbols). Two microcosms (M1 and M2) received similar microbial inocula. No *Pseudomonas* sp. strain B13(FR1) was detected in the uninoculated microcosm. 4CB was added to a final concentration of 10 mM at day 13. Circles, microcosm M1; triangles, microcosm M2. Error bars indicate standard errors of the means.

(iii) Thermal denaturation of DNA. Small samples (100 to 300 μl) of the DNA extracts were dialyzed against 0.1× SSC (1× SSC consists of 0.15 M sodium chloride and 0.015 M sodium citrate) using Mikro-Collodion Bags (Sartorius AG, Göttingen, Germany) as dialyzing tubes. Each DNA solution (60 μl, 0.5 to 1.0 absorbance units) was melted in a 5-carat cuvette (Beckman Instruments, Inc., Fullerton, Calif.) sealed with Nujol mineral oil (Perkin-Elmer Corp., Norwalk, Conn.) by using a Beckman DU-70 spectrophotometer (260 nm) equipped with a thermoprogrammer (Ramcon A/S, Allerød, Denmark). The rate of heating was 1.0°C/min, and data were collected every 6 s.

Calculations. The first derivative of the optical density versus temperature [increase in absorbance/temperature interval (6 s)] was calculated and smoothed by 20% Fast Fourier Transform filtering (Peakfit, Jandel Scientific, Erkrath, Germany).

Calculations of percent G+C contents from melting temperature (T_m) are based on the method described by Owen and Pitcher (38).

RESULTS

Plate counts of *Pseudomonas* sp. strain B13(FR1). The number of CFU of *Pseudomonas* sp. strain B13(FR1) decreased rapidly in the water column of the inoculated microcosms from 10^6 CFU ml^{-1} immediately after the release and was undetectable within 2 to 3 days (Fig. 1A). No colonies were detected from the uninoculated microcosm.

In the sediment, 5×10^4 CFU of *Pseudomonas* sp. strain B13(FR1) g of $sediment^{-1}$ was recovered 1 day after the release of cells. By day 13, the number of CFU had declined to ca. 10^2 cells g of $sediment^{-1}$ (Fig. 1B).

The total number of bacteria in the water was ca. 4×10^6 cells ml^{-1}. The amendment with 10 mM 4CB caused a decrease of the total bacteria to 1.5×10^6 cells ml^{-1}.

Quantitation of *Pseudomonas* sp. strain B13(FR1) by PCR. Amplification was specific for *Pseudomonas* sp. strain B13(FR1) by using the 72°C annealing temperature. No amplification products were observed in the uninoculated control microcosm.

When 10 or more *Pseudomonas* sp. strain B13(FR1) cells ml^{-1} were present in the water samples, equivalent to 400 starting copies per PCR, the B13 fragment and the internal standard were detected on ethidium bromide-stained agarose gels, and the following equation was used to quantitate cells: log of the ratio of B13 fragment to internal standard = $0.628x - 1.493$ ($r^2 = 0.986$). A total of 0.1 cell (4 starting copies per PCR) to 10 cells ml^{-1} were detected on Southern blots and quantitated by using the following equation: log of the ratio of B13 fragment to internal standard = $0.308x - 0.582$ ($r^2 = 0.996$) (31). In sediment, with 40 PCR cycles the equation was as follows: log of the ratio of B13 fragment to internal standard = $0.520x - 2.655$ ($r^2 = 0.912$); 1.2×10^3 cells g of $sediment^{-1}$ (dry weight), equivalent to 8 starting copies per PCR, could be detected.

The number of *Pseudomonas* sp. strain B13(FR1) cells detected by cPCR in the water decreased during the first 2 days after the release, from ca. 10^6 cells to 10^2 cells ml^{-1} (Fig. 1A). From day 2 onward, the population slowly declined to 10 to 30 cells ml^{-1} on day 13. After the amendment with 4CB on day 13, the number of *Pseudomonas* sp. strain B13(FR1) cells increased exponentially, and a 100-fold increase in cell numbers was seen after 7 days.

In the sediment, cPCR detected ca. 3×10^5 *Pseudomonas* sp. strain B13(FR1) cells g of $sediment^{-1}$ (dry weight) 1 day after the release. The number of cells remained constant until day 13, after which a small decrease was seen (Fig. 1B).

Relation between rRNA content and growth rate for *Pseudomonas* sp. strain B13(FR1). The mean fluorescence intensity of the cells as well as the cell size was highly dependent on the growth rate. Therefore, it was necessary to divide the total light emission per cell by the cell area in order to calculate the mean intensity per cell. At each growth rate, 15 cells were quantified from three different digital images, and the background was subtracted for each image. The mean fluorescence intensity per cell showed a sevenfold decrease in intensity for the growth rate range investigated, with the largest decrease at rates between 1.25 and 0.25 h^{-1}. As a control, total RNA was determined chemically by AO staining. The fluorescence signal from AO staining showed a sixfold decrease for the growth rates investigated, and the curve followed the curve when the rRNA content was determined with the rRNA-targeting probes (Fig. 2).

Detection of *Pseudomonas* sp. strain B13(FR1) in microcosms. *Pseudomonas* sp. strain B13(FR1) was detected in microcosms throughout the experimental period by in situ hybridization. Because of autofluorescence and unspecific probe binding, it was sometimes difficult to detect the specific signal from *Pseudomonas* sp. strain B13(FR1) cells when the ordinary rhodamine filter set was used. The inclusion of a narrow-bandpass filter used together with the ordinary rhodamine filter set minimized the problems of autofluorescence. The autofluorescence has a broad unspecific excitation spectrum, while the specific signal has a narrow excitation profile (42). The narrow-bandpass filter excluded up to 90% of the light but discriminated between the autofluorescence from contaminating material and the specific signal from the Ps-B13 probe by allowing light in the 585- to 595-nm range to pass through. This reduced most of the autofluorescence and enhanced visualization of the probe labeling.

A standard curve correlating rRNA content with the growth rate was made with the Ps-B13 probe labeled with rhodamine. The narrow-bandpass filter together with the rhodamine filter

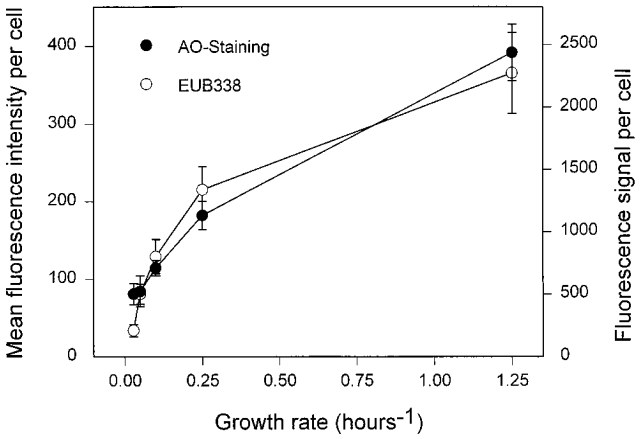

FIG. 2. Mean fluorescence intensity per cell versus the growth rate of *Pseudomonas* sp. strain B13(FR1) inferred by whole-cell hybridization and CCD-enhanced microscopy. The general bacterial rRNA-targeting probe EUB338 was used. The total RNA content was determined by AO staining and CCD-enhanced microscopy. Error bars indicate standard deviations.

set for this standard curve was used. The ribosomal content of *Pseudomonas* sp. strain B13(FR1) 1 hour after the release corresponded to a generation time of 2 h (Fig. 3). At days 1 and 2, the ribosomal contents corresponded to a generation time of approximately 6 h. The ribosomal contents at days 5, 9, 13, and 20 were below the range of the standard curve. The generation time was more than 25 h, or, alternatively, the cells were starved. One day after the amendment with 4CB, the ribosomal content corresponded to a generation time of 8 h, and on day 15 the corresponding generation time was approximately 5 h.

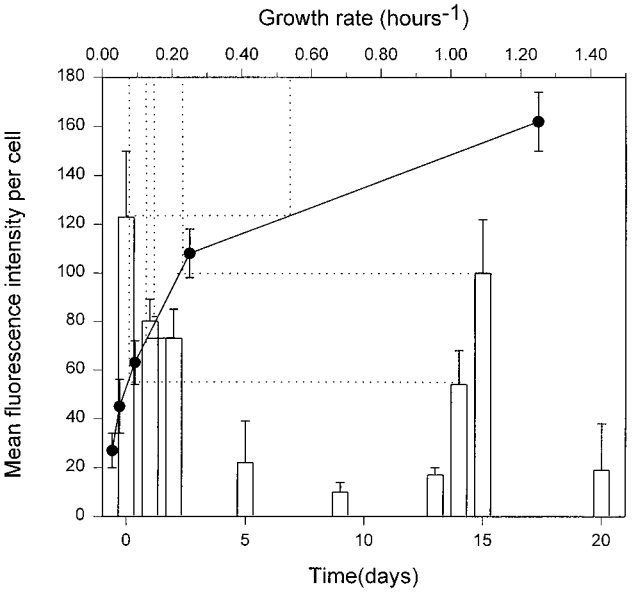

FIG. 3. Standard curve correlating rRNA content with growth rate for *Pseudomonas* sp. strain B13(FR1) obtained by whole-cell hybridization with the Ps-B13 probe of cells growing at different growth rates (shown at the upper *x* axis). The bars show the measurements of the mean ribosomal contents in single cells of *Pseudomonas* sp. strain B13(FR1) from samples obtained from microcosms M1 and M2 at the days indicated on the lower *x* axis. Error bars indicate standard deviations. A narrow-bandpass filter was used in combination with the ordinary rhodamine filter set on the microscope.

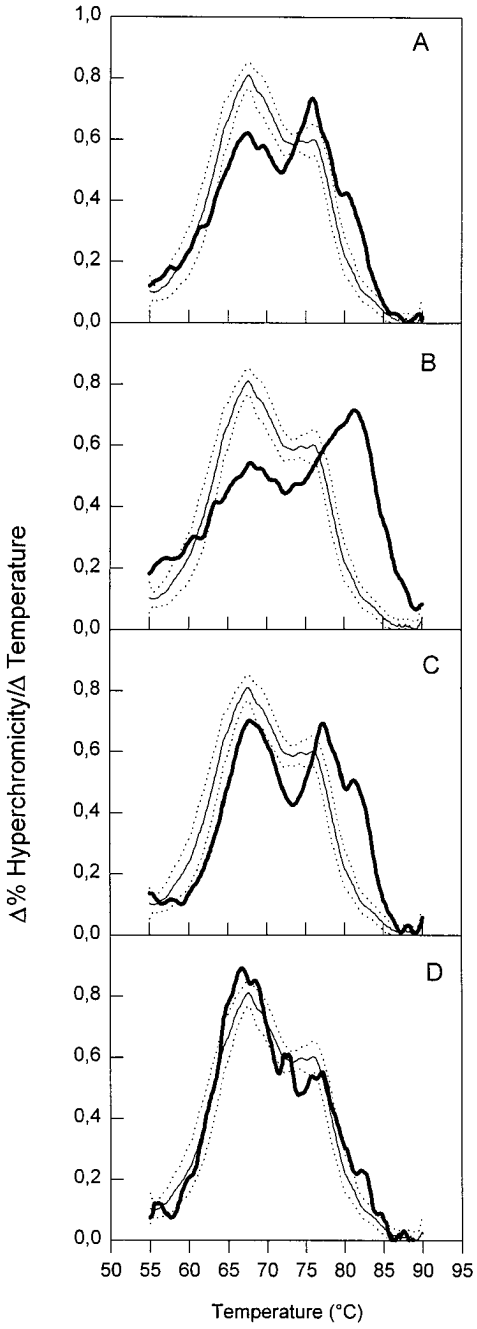

FIG. 4. Melting profiles of DNA extracted from bacterioplankton from Roskilde Fjord (thin lines [means of 10 samples taken during 1993, with 95% confidence intervals given]) and of microcosms (thick lines). (A) Day 0, prior to release of *Pseudomonas* sp. strain B13(FR1) (means of microcosms M1 to M3); (B) day 0, after release of *Pseudomonas* sp. strain B13(FR1) (means of microcosms M1 and M2); (C) day 1 (means of microcosms M1 and M2); (D) day 2 (means of microcosms M1 and M2).

Effects on the indigenous bacterioplankton. The melting profile of DNA from Roskilde Fjord displayed the same pattern through the year (Fig. 4), with a maximum in the denaturation rate at ca. 67°C (≈33% G+C) and a second maximum or shoulder at ca. 76°C (≈52% G+C), while only little DNA denatured above 80°C (≈60% G+C).

The melting profile of DNA from the microcosms before the

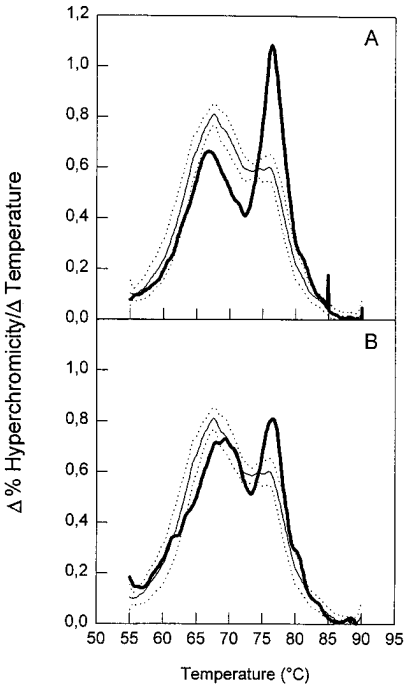

FIG. 5. Melting profiles of DNA extracted from bacterioplankton from Roskilde Fjord (thin lines [means of 10 samples taken during 1993, with 95% confidence intervals given]) and of microcosms (thick lines) after the amendment with 4CB. (A) Day 2 after amendment (means of microcosms M1 to M3); (B) day 7 after amendment (means of microcosms M1 to M3).

release of *Pseudomonas* sp. strain B13(FR1) deviated from this pattern, since the most pronounced maximum was found at 77°C (≈54% G+C), and a significant amount of denaturation occurred at 81°C (≈62% G+C) (Fig. 4A).

Inoculation of the microcosms with *Pseudomonas* sp. strain B13(FR1) caused the occurrence of a very pronounced peak at 83°C (≈66% G+C) (Fig. 4B). This peak corresponded to the T_m for *Pseudomonas* sp. strain B13(FR1) (82°C [≈64% G+C]). Subsequently, this maximum decreased and was only just recognizable 2 days after the release (Fig. 4C and D). During the same 2 days, the remaining part of the melting profile gradually resembled the typical pattern found in the Fjord, except for a small maximum at 72°C (≈43% G+C), 2 days after the inoculation. A similar maximum was never observed with DNA from the Fjord. DNA from the control microcosm also gradually reverted to the typical pattern found in the Fjord (results not shown), but no maximum occurred at 72°C.

During the next 11 days (samples taken 5 and 9 days after the release), the melting profiles from all three microcosms resembled the profile from the Fjord; however, the secondary maximum at ca. 76°C was a little sharper and more pronounced than that normally seen in the Fjord (data not shown).

Two days after the amendment with 4CB (Fig. 5A), the DNA melting profile had a steep, dominating maximum at 77°C (≈54% G+C) and a smaller but broader maximum at 66°C (≈31% G+C). In the following 5 days (Fig. 5B), the 77°C maximum diminished, while the second maximum moved to 68°C (≈35% G+C).

DISCUSSION

Quantitation by cPCR. The detection limit for *Pseudomonas* sp. strain B13(FR1) by cPCR in water was lower than that in sediment. By filtration, bacteria can be concentrated in water samples, without concentrating contaminants, which are inhibitory to the amplification process. Therefore, DNA extracted from large samples can be amplified. Sediment DNA extracts could be amplified only after dilution of the DNA, indicating the presence of humic compounds inhibiting the reaction (52). Inclusion of the single-stranded DNA-binding protein encoded by gene *32* of bacteriophage T4 increased the amount of DNA extract that could be amplified by a factor 10. A total of 40 PCR cycles produced sufficient B13 fragment from 8 starting copies to be detected on ethidium bromide-stained gels, and hybridization did not increase detection sensitivity. A total of 30 PCR cycles were optimal for the water samples, since more cycles produced a DNA smear blurring the B13 fragment (31). However, 30 PCR cycles produced too little B13 fragment to be detected on ethidium bromide-stained gels from less than 400 starting copies. Hybridization increased the detectable amount of B13 fragment by 100-fold, enabling detection of 4 starting copies.

In situ hybridization. The relationship between RNA content and growth rate appears to be universal among bacteria; however, the correlation varies among microorganisms and can be described as parallel lines with different intercepts but a common slope (28). Therefore, standard curves that correlate the mean fluorescence intensity from the rRNA-targeting probe in single cells with the growth rate for the experimental microorganism have to be made.

Determination of the total RNA content by AO staining followed the determination of rRNA content by using the rRNA-targeting probe. The differential fluorescence of AO staining is related to the growth rate (3) and is thus a control of the hybridization when it is used for the same strain under identical conditions.

Hybridization with the non-EUB338 probe, which is unable to bind specifically to rRNA, confirmed that unspecific staining was low and independent of growth rate and starvation.

The standard deviation of the whole-cell hybridization measurements reflects biological variation as well as the error associated with defining the cell boundary by manually circumscribing the cells, which tends to overestimate the sizes of very bright objects and underestimate the sizes of dimmer objects (49).

Survival and activity in microcosms. The numbers of *Pseudomonas* sp. strain B13(FR1) cells detected in the two parallel microcosms corresponded well. Within 2 days, both the number of CFU and the number of cells quantitated by cPCR decreased by approximately 4 log units. A rapid initial decline in cell numbers that is followed by a period with little or no decline is typical of bacteria being grazed by protozoa (1). However, the ability to withstand intensive grazing is related to the growth rate of the released cells (19, 22). The growth rate of *Pseudomonas* sp. strain B13(FR1) decreased after release into the microcosms to less than 0.04 h^{-1}. Growth rates in marine environments vary with the trophic state (35) but are typically less than 0.02 h^{-1} (12, 54, 55). Thus, the rapid decrease of the growth rate was the physiological response of the cells to the change from a very nutrient-rich culture medium to the more oligotrophic conditions of natural ecosystems.

Two days after the release, *Pseudomonas* sp. strain B13 (FR1) was no longer recoverable on plates, although PCR continued to detect cells. Nonculturable cells have been detected by PCR targeting of genomic DNA (5, 7), and possible nonculturable cells of *Pseudomonas* sp. strain B13 were detected in aquifer material (51). The continued detection by cPCR in our experiment was not an artifact due to extracellu-

lar DNA from lysed cells in the water, since the DNA extraction procedure was based on collecting bacteria on membrane filters, which allows macromolecules to pass. Further, *Pseudomonas* sp. strain B13(FR1) was detected throughout the entire period by in situ hybridizations with the Ps-B13 probe, which detects only intact ribosome-containing cells.

After the initial decline of the growth rate, it is possible that the cells entered a starvation-survival state (36). However, this cannot be deduced from the measures of cellular rRNA, since starved cells maintain a pool of ribosomes (14, 18). Alternatively, the cells may have been growing with a generation time longer than 25 h.

The 4CB amendment caused a rapid increase in the growth rate and the number of *Pseudomonas* sp. strain B13(FR1) cells enumerated by cPCR, suggesting that the cells were carbon limited before the amendment. The subsequent growth rate decrease may have been due to limiting concentrations of other nutrients, e.g., phosphate and nitrogen. The culturability of *Pseudomonas* sp. strain B13(FR1) was not restored within 2 days after the amendment.

Pseudomonas sp. strain B1(FR1) colonized the sediment and retained culturability much longer in the sediment. Successful colonization of freshwater sediments from the overlying water column by *Pseudomonas* sp. strain B13 FR1(pFRC20P) was reported by Pipke et al. (40), and in aquifer material, culturable *Pseudomonas* sp. strain B13 cells could be detected after 14.5 months (51). No rapid decline of the population was seen, suggesting that grazing played a minor role in the sediment. The sediment may provide an environment with higher nutrient availability and better protection from predators than the water (1, 22). A fraction of the higher cell numbers detected by cPCR in the sediment may be due to extracellular DNA which was released from lysed cells. Extracellular DNA is coextracted by direct lysis methods (37) and persists in some environments for weeks (43). The numbers of *Pseudomonas* sp. strain B13 (FR1) cells in the sediment detected by cPCR decreased slightly after the amendment with 4CB. A possible explanation may be the accumulation of 4CB in the sediment to a toxic concentration, since 4CB concentrations of 16 mM or more have a detrimental effect on this strain (data not shown).

Effects on the indigenous bacterioplankton. When plotted in the first derivative form, the thermal melting profile of native DNA from a single bacterial strain, exhibits a Gaussian distribution, and the maximum is an estimate of the T_m (38). When the DNA originates from several bacterial strains, a curve of the first derivative of the thermal melting profile theoretically consists of several overlapping Gaussian distributions, which manifest themselves as several maxima and shoulders on the curve. Since the melting profile of DNA from Roskilde Fjord during the year had a maximum of approximately 67°C and a secondary maximum of approximately 76°C, it is reasonable to conclude that the near-surface bacterioplankton was dominated by bacteria with G+C contents of between 30 and 35% and was subdominated by other bacteria with G+C contents of between 50 and 55%, while few bacteria with a G+C content of more than 60% were present.

The confinement of the water in the microcosms caused a shift in the bacterial community structure, since bacteria with a G+C content of between 50 and 55% and with a G+C content of greater than 60% were more abundant after 2 days of confinement. The effect was transient. This is in accordance with Leser (32), who found a higher similarity between bacterioplankton in microcosms and that in a lake ecosystem after 4 days of confinement than after 2 days. He concluded that this could be explained by a transient effect of handling the water during the microcosm-filling procedure, which imposed selection of certain bacterial populations.

The release of *Pseudomonas* sp. strain B13(FR1) into the microcosms resulted in the occurrence of a sharp peak in the DNA melting profiles at a temperature corresponding to the T_m of *Pseudomonas* sp. strain B13(FR1). This peak was apparent during the next 2 days, suggesting that *Pseudomonas* sp. strain B13(FR1) contributed significantly to the melting profile until about 10^3 cells ml^{-1} were present. The release of *Pseudomonas* sp. strain B13(FR1) had no lasting effect on the indigenous bacterioplankton, since the melting profiles of DNAs from the microcosms after a few days resembled each other and that of the Fjord. However, 2 days after the release, a peak appeared at 72°C in the inoculated microcosms. A similar peak was also observed the day after the amendment with a small amount of Luria-Bertani broth in experimental enclosures placed in Roskilde Fjord (23). Hence, this peak might appear as a consequence of the growth of a specific population responding to the nutrient released during the decline of *Pseudomonas* sp. strain B13(FR1). This would be in accordance with Höfle (26), who found that the effects of the release of bacteria on the community structure of bacterioplankton could be attributed to the addition of nutrients.

The amendment of the microcosms with 4CB caused a dominans after 2 days of bacterial populations with a G+C content of approximately 54%. This could be a consequence either of differential survival of these populations, since only about 40% of the bacteria survived the amendment with 4CB, or of growth of a specific population, which tolerated 4CB. During the subsequent 5 days, populations with a G+C content of about 35% proliferated, while the populations with a G+C content of about 54% declined, as the total number of bacteria remained relatively constant.

In conclusion, by using highly sensitive molecular techniques for monitoring cell numbers and activity, we could demonstrate that a genetically engineered bacterial strain introduced into microcosms was able to sustain a small population, which was undetectable by traditional methods, and that this population responded to selective nutrient enrichment by increasing its growth rate, resulting in increased cell numbers. The release of this strain had no pronounced effect on the indigenous bacterioplankton; however, the bacterial community was affected by the handling of the water during microcosm filling and by the amendment with 4CB.

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