APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1993, p. 1354–1360
0099-2240/93/051354-07$02.00/0

Vol. 59, No. 5

Use of rRNA Fluorescence In Situ Hybridization for Measuring the Activity of Single Cells in Young and Established Biofilms

LARS K. POULSEN,[1]† GWYN BALLARD,[2] AND DAVID A. STAHL[1,3]*

Departments of Veterinary Pathobiology[1] and Microbiology,[3] University of Illinois, Champaign-Urbana, 2001 South Lincoln Avenue, Urbana, Illinois 61801, and Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510-8005[2]

Received 17 December 1992/Accepted 17 February 1993

We describe the in situ use of rRNA-targeted fluorescent hybridization probes in combination with digital microscopy to quantify the cellular content of ribosomes in relationship to the growth rate of single cells of a specific population of sulfate-reducing bacteria in multispecies anaerobic biofilms. Using this technique, we inferred that this population was growing with an average generation time of 35 h in a young biofilm, whereas the doubling time in an established biofilm was significantly longer. Conventional chemical determinations of the RNA, DNA, and protein contents of this culture at different growth rates were also carried out, and the resulting data were compared with the rRNA fluorescence in situ hybridization data.

The presence of specific genes or microorganisms in the environment can be inferred by using direct biochemical or molecular measures (e.g., lipid analysis or DNA or RNA sequence analysis) (22). However, such measures provide little information concerning the metabolic activity of specific organisms making up different environmental populations or the responses of these organisms to changing biotic and abiotic conditions. Thus, the development of methods to evaluate both single-cell identity and activity should offer a useful complement to existing direct detection methods. In this study we evaluated an approach to the simultaneous measurement of single-cell identity and growth rate in multispecies biofilm communities. Both of these measures are based upon hybridization of tagged oligonucleotide probes to rRNAs.

The use of rRNA sequence divergence to infer phylogenetic relationships and as the basis for developing determinative hybridization probes is now well established (1–3, 12, 24). An increasingly popular method is the use of fluorescent-dye-labeled oligonucleotides complementary to rRNAs for the visualization of single cells with fluorescence microscopy (2, 3, 14, 21). In principle, cellular ribosome content can also be inferred from the results of hybridization of fluorescent probes to cellular rRNA.

The correlation between cellular ribosome (rRNA) content and growth rate was one of the earliest and most fundamental observations in microbial physiology (17). Thus, fluorescent-dye-tagged oligonucleotide probes might be used as a basis for estimating the growth rates of single cells in a variety of settings. Delong et al. (5) demonstrated the application of this general approach to the study of cells in pure culture. However, since oligonucleotide probes can be designed to hybridize to specific species of microorganisms, this measure could in principle also be used to evaluate the growth rates of specific populations within complex microbial communities. In this study, we used this approach to infer the growth rate of single cells making up a population of sulfate-reducing bacteria in multispecies biofilms.

Biofilms were established in an anaerobic fixed-bed bioreactor system. Similar systems have been used as model systems to study biofilm processes (15) and more recently to evaluate molecular techniques for the study of microbial population ecology (3). The study reactor was established previously from a groundwater inoculum and was maintained on a glucose-based medium containing sulfate as a terminal electron acceptor. Previous studies have shown that this system maintains a stable and complex community with associated populations of sulfate-reducing bacteria (3, 14). A sulfate-reducing bacterium previously isolated from this reactor was characterized in pure culture as a prelude to the studies described below (17). We report the relationships among cellular composition, rRNA fluorescence in situ hybridization signal intensity, and the growth rate of this microorganism. For these studies, a cooled, slow-scan, charge-coupled device camera attached to an epifluorescence microscope was used to capture images of hybridization signals from single cells (23). Analysis of digitized images was subsequently used to quantify cell size and fluorescence intensity both in batch culture and in multispecies biofilms. The results of this study indicated that this approach should be useful for directly inferring the growth rates of microorganisms, even very slowly growing microorganisms, in the environment.

MATERIALS AND METHODS

Organisms. The sulfate-reducing bacterium strain PT2 was isolated previously from a sulfidogenic bioreactor (8) (see below). This isolate exhibits 94% 16S rRNA sequence similarity with *Desulfovibrio vulgaris* and a similar range of substrates and electron-accepting species (8). *Escherichia coli* MC4100 is a derivative of *E. coli* K-12 (19).

Media. The composition of the basic medium used for the pure-culture studies of strain PT2 has been described previously (8). Lactate, malate, fumarate, or formate was used at a concentration of 20 mM. Acetate was used at a concentration of 10 mM. The bioreactor was fed a glucose-sulfate-

* Corresponding author.
† Present address: Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark.

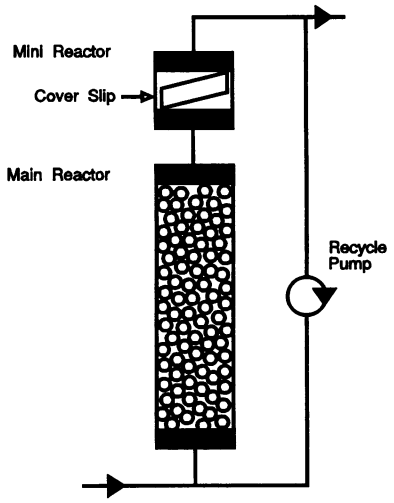

FIG. 1. Schematic drawing of the minireactor and the main bioreactor. The arrows indicate flow directions.

minerals medium as previously described (12). *E. coli* was grown in M9 medium containing 0.2% glucose (18). The optical densities of cultures were determined at 600 nm with a Spectronic 20 colorimeter (Milton Roy). Cell numbers were determined with a bacterium-counting chamber (Hausser Scientific Partners, Horsham, Pa.) following dilution of the culture with an equal volume of 0.1 M HCl.

Growth rate determination. The growth rate studies were conducted at the bioreactor operating temperature (23°C). Prior to determination of the culture composition at each specific growth rate, strain PT2 was grown for 15 to 20 generations to ensure that the culture was in balanced growth. The growth rate was determined in batch culture by measuring the increases in both turbidity and protein content. Cultures were harvested at an optical density of approximately 0.1 (mid-log phase) for determinations of cell number and RNA, DNA, and protein contents. At this time a portion of the culture was fixed for subsequent hybridization of cells with the fluorescent probes.

Bioreactor sampling. The fixed-bed bioreactor used in this study was maintained under anaerobic conditions at 23°C as previously described (3, 15). This reactor had been inoculated previously (August 1988) with an inoculum derived from groundwater and at the time of the study contained a stable and complex microbial community (3, 14). Two types of biofilm were sampled, biofilm in the early stages of development (designated young biofilm) and biofilm derived from established material (designated old biofilm). The young biofilm (one to three layers of cells) was recovered on glass coverslips placed for short periods of time in a vertical position within a minireactor. As shown in Fig. 1, the minireactor was a short column that had the same diameter as the main reactor and was connected to the outlet of the main reactor. This configuration ensured that the coverslips experienced the same medium composition and flow as material within the main reactor. The old biofilm was obtained by removing five beads (diameter, 3 mm), with attached biofilm and floc, from the main reactor. This sample was placed in a glass test tube (12 by 100 mm) containing 3 ml of reactor medium and disaggregated by sonication for approximately 10 min in a model 1200 sonicator (Branson Cleaning Equipment Co., Shelton, Conn.) containing ice water. Released cells were inspected microscopically with increasing time of sonication to monitor disaggregation and possible cellular disruption. Each young biofilm was fixed without removal from the coverslip as previously described (12). Both the attached and disaggregated biofilms were fixed prior to hybridization as described below.

RNA, DNA, and protein determinations. For the batch culture analyses we used 250-ml cultures grown anaerobically with gentle shaking. Immediately prior to harvesting, samples were removed for nucleic acid and protein analyses, optical density measurement, direct cell counting, and hybridization. The samples used for hybridization were immediately fixed (see below). RNA and DNA contents were determined with 0.5- and 0.1-ml samples, respectively, by using the orcinol and diphenylamine assays as previously described (7). Purified 16S and 23S rRNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used as an RNA standard (assuming that an optical density at 260 nm of 1 was equivalent to 40 μg/ml), and chromosomal DNA isolated from *E. coli* (Sigma Chemical Co., St. Louis, Mo.) was used as a DNA standard (assuming that an optical density at 260 nm of 1 was equivalent to 50 μg/ml). The Coomassie brilliant blue G-250 assay (Bio-Rad, Richmond, Calif.) was used to estimate protein content according to the manufacturer's instructions, using bovine serum albumin as a standard (Sigma). The assay was done in triplicate (three culture samples) for each time point.

Oligonucleotide probes. Oligonucleotide probes were synthesized with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) operated by workers at the University of Illinois Biotechnology Center. An aminohexyl linker (Aminolink 2; Applied Biosystems) was attached at the 5′ terminus by using a standard DNA synthesis cycle. The specific probe for strain PT2 (PT2 probe; 5′-TCTCCCGAACTCA AGTCCA-3′) has been described previously (3, 8) and is complementary to a region corresponding to positions 647 to 665 in the *E. coli* 16S rRNA. The general probe for the bacterial domain (5′-GCTGCCTCCCGTAGGAGT-3′), designated EUB338, has also been described previously (20). Both probes were labeled with tetramethylrhodamine isothiocyanate (Research Organics, Cleveland, Ohio) and purified as previously described (20). The PT2 probe was extended by poly(T) tailing, using terminal deoxynucleotidyl transferase (United States Biochemical Corp., Cleveland, Ohio). The reaction mixture consisted of 90 μg of oligonucleotide, an 80-fold molar excess of TTP, 100 mM sodium cacodylate (pH 7.2), 0.2 mM 2-mercaptoethanol, and 2 mM $CoCl_2$. Following addition of 25 U of enzyme, the mixture was incubated for 2.5 h at 37°C, and unincorporated nucleotides were removed by passage of the reaction mixture through a G-25 Spincolumn (5′-3′, West Chester, Pa.). The probe was then labeled with tetramethylrhodamine isothiocyanate and fractionated by size on a polyacrylamide gel. Regions of the gels containing different size fractions were excised, eluted, and purified as previously described (1). The average length of each extended probe fraction was estimated by comigration on a DNA sequencing gel.

Hybridization of whole cells and biofilms. Prior to hybridization, cultured cells, or biofilms were fixed in 4% paraformaldehyde as previously described (3). For hybridization of single cells (from a culture or disaggregated biofilm), cells were bound to gelatin-coated slides and dried by sequential washes in 50, 80, and 100% ethanol (3 min each) (12). Following the ethanol series washes, 9 μl of hybridization mixture (30% formamide, 0.9 M NaCl, 20 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate [SDS]) contain-

ing 25 ng of probe was added to each sample position on the slide. The cells were hybridized for 4 h at 37°C in a moisture chamber (1, 20), washed twice for 25 min at 37°C in 20 μl of hybridization solution (without probe), and then rinsed with water. The young biofilms were examined by breaking off a small piece of coverslip with associated biofilm and using the same hybridization conditions as described above. The hybridized specimens were visualized by epifluorescence microscopy as previously described (12).

DAPI staining. The cells were first immobilized on gelatin-coated slides and then stained with DAPI (4′,6-diamidino-2-phenylindole) for 5 min by adding 30 μl of a 38.5-μg/ml DAPI solution in 30% formamide–0.9% NaCl–20 mM Tris-HCl (pH 7.2)–0.1% SDS to the hybridization well. The cells were washed twice in a solution of the same composition without DAPI.

Image analysis and photomicrography. Two epifluorescence microscopes were used, an Olympus BH2 microscope and a Carl Zeiss Axioskope-20 microscope attached to a 16-bit cooled charge-coupled device camera (model CH250; Photometrics, Tucson, Ariz.). For images captured with a ×100 objective, 1 pixel corresponded to 0.042 μm of the image field. A Macintosh computer was used to run IPlab image analysis software (SU2, 14 bit). The edge of a cell was manually circumscribed by using a pointing device, and the resulting data were used to determine cell size and integrated fluorescence intensity. Photomicrography was performed as previously described (1).

RESULTS AND DISCUSSION

Determination of growth-related cellular parameters. Strain PT2 is a recent isolate that was obtained from a bioreactor and initially exhibited significant wall growth in culture tubes. Since wall growth complicated physiological experiments, a variant of strain PT2 which only grew in suspension was isolated. This variant was selected for by sequential dilution of batch cultures for about 60 generations. In order to characterize changing cellular composition associated with changing growth rate, strain PT2 was grown in five different media which resulted in doubling times ranging from 5.75 to 37.5 h. Sulfate was provided as an electron acceptor in all media (see Materials and Methods). Inclusion of the electron donors lactate, malate, fumarate, formate plus acetate, and formate resulted in generation times of 5.75, 7.5, 8.25, 20, and 37.5 h, respectively.

Measurements of the mean cellular RNA content determined chemically demonstrated that there was a four- to fivefold change in content per cell between generation times of 5.75 and 37.5 h (Fig. 2A), corresponding to growth rates of 0.174 and 0.027 doubling per h, respectively (growth rate = $1/g$, where g is the generation time in hours). The relationship between ribosome content and growth rate was approximately linear as previously observed for *E. coli*, *Salmonella typhimurium*, and *Aerobacter aerogenes* (4, 16). Assuming that 80% of the total RNA is rRNA (11), the ribosome content was inferred to vary from about 35,000 to 8,000 ribosomes per cell over this range. The greatest variation in RNA content was observed between generation times of 5.75 and 20 h (growth rates, 0.174 and 0.05 doubling per h, respectively), with a lesser change between 20 to 37 h.

The growth rate-related change in DNA content is also shown in Fig. 2A. At the maximum growth rate evaluated (0.174 doubling per h, corresponding to a 5.75-h generation time), there was approximately 5×10^{-8} μg of DNA per cell. A 1.8-fold change in DNA content per cell was associated

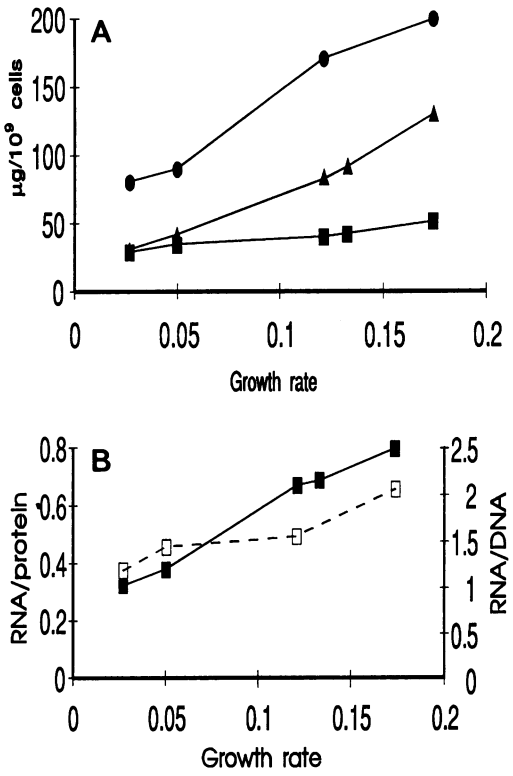

FIG. 2. Relationship between the growth rate and the cellular contents of RNA, DNA, and protein for strain PT2. (A) Total RNA content per cell (▲), total DNA content per cell (■), and total protein content per cell (●) at five different growth rates. (B) The RNA/DNA (■) and RNA/protein (□) ratios at five different growth rates.

with a change in generation time from 5.75 to 37.5 h. Thus, if the molecular weight of the strain PT2 genome is comparable to the estimate of 1.14×10^9 reported at the stationary phase of batch growth (13) for *D. vulgaris*, an organism to which this isolate is related, a cell growing at the maximum growth rate would contain approximately 26 chromosomes. Although this number is greater than the estimate of 4 chromosomes per cell for *D. vulgaris*, it is comparable to the estimate of 17.2 chromosomes per cell for *Desulfovibrio gigas* growing with a 33-h generation time (13). However, given the uncertainty of these earlier genome size determinations and the possible contribution of extrachromosomal DNA, a reevaluation of previous size estimates should be considered.

In addition to the chemical determination of DNA content, digital imaging was used to quantify the DNA content of DAPI-stained cells (see Materials and Methods). The results of this quantification were comparable to the results of the chemical determination and showed a 2.1-fold change in DNA content over the range of growth rates examined (data not shown).

The protein content of strain PT2 was about 2×10^{-7} μg of protein per cell at a 5.75-h generation time (Fig. 2A). For the observed range of generation times (5.75 to 37.5 h), the protein content of strain PT2 cells varied about threefold.

Relative changes in cellular composition were also evaluated. The relationship between RNA content and either protein or DNA content (RNA/protein ratio and RNA/DNA

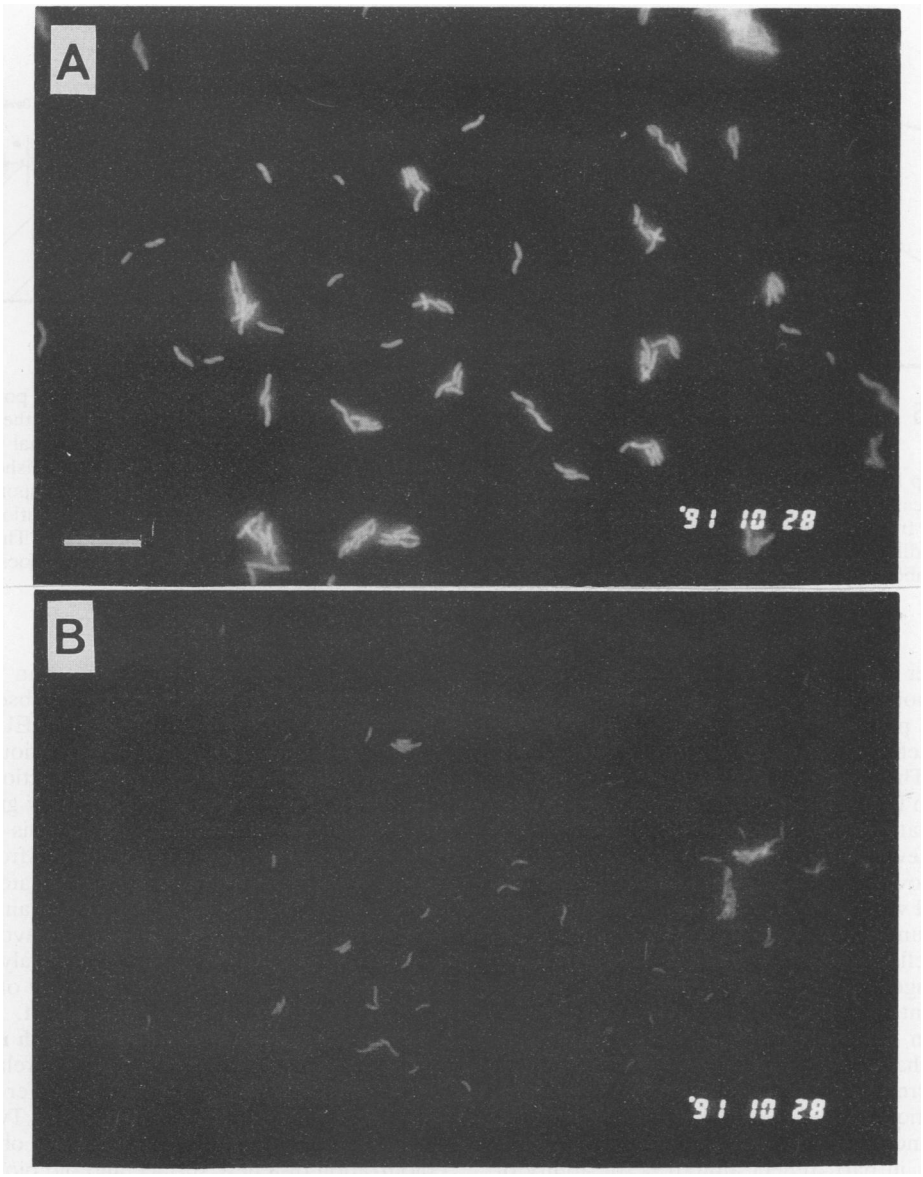

FIG. 3. Microscopic comparison of strain PT2 growing at 6-h (A) and 37-h (B) generation times. Cells were hybridized with the PT2 probe and visualized by epifluorescence microscopy (see Materials and Methods). Bar = 10 μm.

ratio) at the growth rates tested is shown in Fig. 2B. With a change in generation time from 5.75 to 37.5 h, the RNA/DNA ratio declined from 2.5 to 1.0 and the RNA/protein ratio declined from 0.65 to 0.38. In contrast, studies of *E. coli* at generation times between 0.4 and 1.6 h demonstrated that the ratio of RNA to DNA ranged from 14.0 to 3.2 and the ratio of RNA to protein ranged from 0.5 to 0.2 (4).

Change in cellular composition accompanying early stages of starvation. A preliminary study of the response of strain PT2 to starvation was also completed. In this study, the RNA and DNA contents of this strain were measured at 6 h after cessation of growth in a lactate-based batch culture. The RNA content determined at this point was 0.5×10^{-7} μg per cell, suggesting that there was an approximately 60% reduction in RNA content during the 6-h period in stationary phase. In contrast, the DNA content (5×10^{-8} μg per cell) remained comparable to the DNA content prior to entry into stationary phase.

Determination of cellular ribosome content by whole-cell hybridization. The relationship between the intensity of the rRNA fluorescence in situ hybridization probe signal and the growth rate in batch culture was determined as a prelude to the multispecies biofilm studies. Although the total hybridization signal per cell was easily determined with the image analysis software, this measurement was unsuitable for comparison with growth rate. This is because cell size varies with the point in the cell cycle. Variations in cell size and ribosome content are apparent in the photomicrographs of strain PT2 growing at 6- and 37-h generation times (Fig. 3). Size variation did not complicate the chemical determination much, since variation in cell size was averaged over the large number of cells present in each sample. However, for the

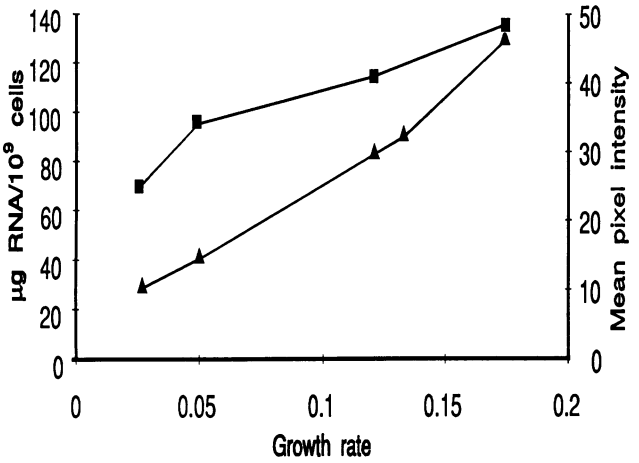

FIG. 4. Relationship between RNA content, inferred by chemical and fluorescence measurements, and growth rate. The total RNA content per cell (▲) is from Fig. 2A. The mean pixel intensity (■) was calculated by dividing the total fluorescence signal per cell by the corresponding number of pixels covering the cell.

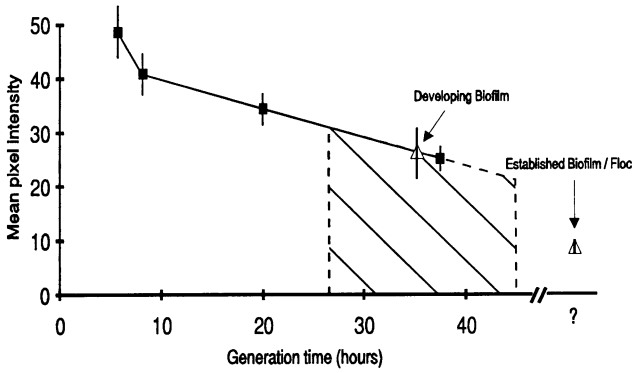

FIG. 5. Inferred growth rates of biofilm populations. The mean pixel intensities (■) are shown along with the standard deviations based on 20 to 25 quantified cells. The signal intensities of cells in young (developing) and steady-state (established) biofilms are indicated (△). The cross-hatched area corresponds to the range of growth rates covered by the standard deviation of 50 cells characterized from the young developing biofilm. The standard deviation of the value for the established biofilm (flocs) was based on the results obtained for nine cells.

much smaller number of cells evaluated by image analysis, it was necessary to normalize fluorescence by dividing the total light emission per cell by the image area (the total number of pixels defining the cell) to give a mean pixel intensity per cell. The mean pixel intensity per cell was determined for strain PT2 at four different growth rates (Fig. 4). At each growth rate, 20 to 25 cells were quantified. A 10 to 20% standard deviation was observed for the signal intensity at each growth rate. This deviation probably reflects both biological variation and the error associated with defining the cell boundaries. The mean pixel intensity per cell for hybridized cells varied by a factor of about 1.9 across the growth rate range and generally corresponded to the cellular RNA content as determined with orcinol (Fig. 4). The greater range in RNA content per cell as determined chemically reflects changes associated with variation in both average cell size (increasing with increasing growth rate) and ribosome concentration.

However, it remained possible that change in fluorescence intensity might also, in part, reflect altered permeability of cells to the probe. To evaluate this, we constructed a set of hybridization probes having increasing lengths by adding a tail of thymidine residues to the PT2-specific probe, using terminal deoxynucleotidyl transferase. Probe-conferred fluorescence intensities, as determined by using five different size fractions of the tailed probe (corresponding to 19 [nonextended], 30 to 70, 70 to 100, 100 to 120, and 120 to 150 nucleotides) were quantified by image analysis. Although the level of nonspecific binding to *E. coli* was slightly elevated when the extended probes were used, the fluorescence intensities of cells hybridized with the probes (evaluated for cultures growing at doubling times of 5.75 and 37.5 h) were comparable (data not shown). These studies indicated that altered permeability did not contribute significantly to the change in fluorescence associated with changing growth rate. In addition, similar fluorescence intensity measurements were obtained by using either the PT2-specific probe or a general probe for the bacteria (EUB338). In aggregate, the results of these experiments support the use of probe hybridization for direct measurement of cellular ribosome content.

A comparison of *E. coli* and strain PT2 growth rate-associated ribosome contents was subsequently made by using the general hybridization probe, EUB338. The results of the orcinol determination had previously indicated that strain PT2 growing at a 5.75-h generation time (23°C) had about the same RNA content as *E. coli* growing at a 60-min doubling time (37°C) (5). Image analysis demonstrated that the total amount of probe fluorescence from *E. coli* growing at a 60-min doubling time was 13% greater than the amount of probe fluorescence measured for strain PT2 growing at a 5.75-h doubling time. This compares favorably with the 8% difference determined by chemical analysis. Thus, the results of this study support the validity of using fluorescent rRNA probes to measure RNA content.

Direct estimation of strain PT2 growth rate in multispecies biofilms. A standard curve (Fig. 4) relating fluorescence intensity to growth rate was used to infer the growth rate of strain PT2 within multispecies biofilms. Two types of biofilm material were compared, young biofilm obtained from initial colonization of a new surface and old biofilm obtained from long established (presumed steady-state) bioreactor material. The young biofilm was obtained by placing a clean glass coverslip in a minireactor coupled to the main reactor (Fig. 1). The coverslip was removed after 7 days, and the biofilm, consisting of one to three layers of cells, was fixed and hybridized with the strain PT2-specific fluorescent probe. The mean pixel intensity of 50 cells was determined and compared with the standard curve (Fig. 5). The cross-hatched area in Fig. 5 corresponds to the range of doubling times encompassed by the standard deviation. This spans generation times of approximately 25 to 45 h.

The inference of growth rate in the established biofilm was based on the mean pixel intensity of 10 cells hybridizing with the PT2-specific probe. One of these cells was found to have a mean pixel intensity corresponding to a doubling time of about 33 h. However, the remaining nine cells fluoresced much less intensely. In fact, the mean pixel intensity for these organisms (Fig. 5) is not represented by the standard curve. The mean values for young and established biofilm cells are significantly different as determined by the t test ($t = 1.13$; df = 58; $P < 0.0001$). Thus, although the sample set

obtained for the established biofilm is relatively small (10 data points), a difference between the two populations is evident. Linear extrapolation of the standard curve to the mean value for the established biofilm cells indicated a doubling time of approximately 70 h. However, this probably is a considerable underestimate since the relationship is almost certainly not linear. Also, the retention time for biomass in the bioreactor has been estimated to be between 2 and 3 months, and the average generation time for organisms within the main reactor may be weeks or months rather than hours or days. Thus, the applicability of this method to the characterization of populations having vastly different growth rates was demonstrated.

Given that extracellular material associated with the biofilm could alter the rate and/or extent of hybridization, hybridization of both young and old biofilms was also evaluated by using the extended probes (see above). The use of the longest probe (ca. 130 nucleotides) resulted in a very high level of background fluorescence, preventing quantification of specific hybridization. Although hybridization with a shorter probe (ca. 80 nucleotides) resulted in slightly elevated levels of background fluorescence, it produced the same mean pixel intensity that was observed following hybridization with the nonextended probe (after background value subtraction). We also compared hybridization of both intact and disaggregated old biofilms. Cell flocs were disaggregated by mild sonication prior to hybridization (see Materials and Methods). No measurable difference in fluorescence intensity was observed (data not shown), indicating that neither sonication nor aggregation measurably altered hybridization.

One limitation to the method described here is that a standard curve is required; cells must be isolated in pure culture prior to their study in the environment. However, the need for a standard curve for each organism might be alleviated in part by using the change in the relative contents of RNA and DNA (RNA/DNA ratio) to estimate the growth rate. The RNA/DNA ratio has been used as a general indicator of growth rate and biomass in the environment (6). This ratio may also be a more useful general value for estimating the relative growth rate of a specific organism. The RNA content and the DNA content could be simultaneously estimated by using fluorescent oligonucleotide probes in combination with DAPI staining. However, additional studies will be needed to establish the character of this relationship for a variety of environmentally relevant organisms.

The timing of chromosome replication with long generation times is also of considerable interest. Although this cell cycle parameter was not directly addressed in this study, the techniques are applicable to its study. For example, work of Skarstad et al. (18) in which flow cytometry was used to quantify the DNA content of slowly growing *E. coli* cultures demonstrated that the B period (the time between cell division and the initiation of a new round of chromosome replication) at a 17-h generation time was 80% of the doubling time (18). Thus, continued studies of PT2 should allow us to quantify DAPI-conferred fluorescence of larger cell numbers and evaluate the use of chemostats for a more systematic study of growth rate-related cellular changes.

Fundamental questions that have yet to be addressed include the comparability of ribosome contents for different organisms growing with the same generation time and the influence of long-term selection in pure culture on this relationship. The influence of temperature also has not been well explored. Studies of *S. typhimurium* during balanced growth demonstrated that size and composition were not influenced by the temperature of cultivation (17). A recent study in which laboratory strains of *E. coli* were compared with more recent "wild-type" isolates demonstrated that laboratory strains grow faster than wild-type cells having approximately the same content of ribosomes. It has been suggested that this reflects a selection for ribosomes having greater efficiency in laboratory strains (10). The variation in ribosome content among the *E. coli* strains examined was about 10% and was no longer evident after prolonged chemostat selection (280 generations) (9, 10).

Although the physiological significance and ecological significance of the observed differences in growth rate-related ribosome content are unclear, one practical consequence is that changing ribosome content should provide a useful tool for evaluating the status o f a great variety of microorganisms growing at a wide range of generation times. One format for the application of this tool to the study of natural microbial communities would be to use change in ribosome content as a basis for evaluating the response of specific populations to altered environments (for example, the addition of alternative substrates or electron acceptors). In addition, heterogeneity within a population (for example, differing activity of single cells associated with increasing depth in a biofilm) might provide useful insights into aspects of substrate and mass transport limitation. For example, in the study reported here, the fluorescence of one cell observed in the established biofilm was significantly more intense than the fluorescence of the other cells observed, corresponding to a doubling time of about 33 h. This may indicate that the established population was composed of cells having a variety of growth rates. Such differences might reflect different positions of cells within the biofilm matrix. For example, cells located near the surface of the biofilm may not be as limited by mass transport of, or competition by surrounding cells for, nutrients. Finally, a more applied application might be the use of this measure to evaluate the growth or activity status of different attached microbial populations associated with corrosion and biodeterioration.

ACKNOWLEDGMENTS

This research was supported by a grant to D.A.S. from the Office of Naval Research. L.K.P. was supported by the Danish Research Center for Microbial Ecology, Danish Technical Research Council, and The Plasmid Foundation.

REFERENCES

1. **Amann, R. I., B. Binder, S. W. Chisholm, R. Olsen, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
3. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
4. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
5. **Delong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for identification of

single cells. Science **243:**1360–1363.
6. **Dortch, Q., T. L. Roberts, J. R. Clayton, Jr., and S. I. Ahmed.** 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. Mar. Ecol. Prog. Ser. **13:**61–71.
7. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 329–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
8. **Kane, M., L. K. Poulsen, and D. A. Stahl.** 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridizing probes designed from environmentally derived 16S RNA sequences. Appl. Environ. Microbiol. **59:**682–686.
9. **Mikkola, R., and C. G. Kurland.** 1991. Evidence for demand-regulation of ribosome accumulation in *E. coli*. Biochimie **73:**1551–1556.
10. **Mikkola, R., and C. G. Kurland.** 1992. Selection of laboratory wild type phenotype from natural isolates in chemostats. Mol. Biol. Evol. **9:**394–402.
11. **Neidhardt, F.** 1987. Chemical composition of *Escherichia coli*, p. 3–6. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaecter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
12. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40:**337–365.
13. **Postgate, J. R., H. M. Kent, R. L. Robson, and J. A. Chesshyre.** 1984. The genomes of *Desulfovibrio gigas* and *D. vulgaris*. J. Gen. Microbiol. **130:**1597–1601.
14. **Raskin, L., and D. A. Stahl.** Unpublished data.
15. **Rittmann, B. E., L. Crawford, C. K. Tuck, and E. Namkung.** 1986. *In situ* determination of kinetic parameters for biofilms: isolation and characterization of oligotrophic biofilms. Biotechnol. Bioeng. **28:**1753–1760.
16. **Rosset, R., J. Julien, and R. Monier.** 1966. Ribonucleic acid composition of bacteria as a function of growth rate. J. Mol. Biol. **18:**308–320.
17. **Schaechter, M., O. Maaloe, and N. O. Kjeldgaard.** 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19:**592–606.
18. **Skarstad, K., H. B. Steen, and E. Boye.** 1983. Cell cycle parameters of slowly growing *Escherichia coli* B/r studied by flow cytometry. J. Bacteriol. **154:**656–662.
19. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Stahl, D. A., and R. I. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.
21. **Stahl, D. A., R. Devereux, R. I. Amann, B. Flesher, C. Lin, and J. Stromley.** 1989. Ribosomal RNA based studies of natural microbial diversity and ecology, p. 669–673. *In* Y. Hattoir, Y. Ishida, Y. Maruyama, R. Y. Morita, and A. Uchida (ed.), Recent advances in microbial ecology. Proceedings of the 5th International Symposium on Microbial Ecology. Japan Scientific Societies Press, Tokyo.
22. **Stahl, D. A., and M. D. Kane.** 1992. Methods of microbial identification, tracking and monitoring of function. Curr. Opinion Biotechnol. **3:**244–252.
23. **Vites, C. L., and M. E. Sieracki.** 1992. Measurement of marine picoplankton cell size by using a cooled, charged-coupled device camera with image-analyzed fluorescence microscopy. Appl. Environ. Microbiol. **58:**584–594.
24. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.