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Quantitation of the population size and metabolic activity of a resin acid-degrading bacterium in activated sludge using slot blot hybridization to measure the rRNA:rDNA ratio

Running title:
Quantitation of population size and metabolic activity

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ABSTRACT

The 16S rRNA:rDNA ratio is a useful parameter for measuring metabolic activity of a selected member of a complex microbial community, as in pulp effluent activated sludge systems. The RNA:DNA ratio of Sphingomonas sp. DhA-33, previously isolated from a sequencing batch reactor treating pulp mill effluent, is positively correlated with its growth rate (μ) under steady-state conditions. DhA-33 was grown in a chemostat with growth rates ranging from 0.04 to 0.15 cell divisions per hour. DhA-33 was also able to degrade dehydroabietic acid in bleached kraft mill effluent (BKME) plus mineral medium in batch culture. We used slot-blot hybridization with radioactively labeled species-specific oligonucleotide probes for 16S rRNA and 16S rDNA to measure rRNA, rDNA, and the RNA:DNA ratio of this strain when in a mixed sludge community. An increase in DhA-33 rDNA indicated growth of DhA-33 within the community. The RNA:DNA ratio of DhA-33 increased sharply during exponential growth and declined as cells entered stationary phase. The RNA:DNA ratio decreased earlier and faster in DhA-33/sludge co-cultures than in DhA-33 pure cultures presumambly due to an earlier depletion of nutrients. The species-specific quantification of the RNA:DNA ratio makes it possible to estimate the metabolic activity of selected members of a microbial community in situ.

INTRODUCTION

All processes used in pulp and paper manufacture require an abundant supply of fresh water. The resulting effluents have been shown to be highly toxic to fish (34) (25). Most of that toxicity has been attributed to resin acids which are natural components of wood which enter the waste water stream during pulping (16). Increasing environmental (28) and economic pressure on pulp and paper mills to limit the amount of water used and discharged have led to the objective of mill closure through containment and re-use of waste water. Recirculating waste water results in the formation of pitch which can be detrimental to paper machines and consists largely of resin acids (8). The accumulation of resin acids and other substances in discharged waste water, in recycled water and in runoff from forest operations (37)must be controlled through some form of treatment.

Biological treatment has long been used to reduce BOD (biological oxygen demand) and toxicity of pulp and paper mill waste water (38). However, the performance of biological treatment systems is variable, depending on waste loading rates and other environmental factors which have the potential to cause stress to the activated sludge. Therefore, a deeper understanding of the ecology of a bioreactor, the significance of resin acid-degrading bacteria within the activated sludge, and their metabolic activities are desired. Classical microbiology techniques have enabled us to isolate and characterize resin acid-degraders (19) (20) (36), but do not enable us to monitor the metabolic activity of these species within a natural community where other organisms share the same activities or where growth may be below detectable levels. Most methods measuring metabolic activity, such as acridine orange activity stain, direct viability counts using nalidixic acid (15), ATP concentration (33), $^{14}CO_2$ emission as a measure of mineralization, or GC/HPLC to measure the disappearance of a pollutant are not specific for individual species. The aim of this research is to develop a molecular approach to measure populations of resin acid-degraders in their natural environment and to quantify their growth rate or metabolic activity.

The molecular approach we developed is based on the observation that the cellular ratio of RNA to DNA increases as growth rate increases. This observation has been shown for E. coli (2), S. typhimurium (30), A. aerogenes (22), more recently for slow-growing marine isolates (13, 14) and for a number of selected resin acid-degrading bacteria (21). The amount of RNA and DNA in the cell is primarily dependent on the rate at which the cell is growing and is affected by the chemical composition of the growth medium only in so far as this affects the growth rate. Far less is known about the effects of physical environmental factors such as temperature on the chemical composition of the cell (6). It was also shown that the RNA content of 'resting' cells was lower than the RNA content of 'log phase' cells in batch cultures of a number of gram positive and gram negative species (11). The amount of cellular DNA is relatively stable and can be exploited to measure the number of cells present; whereas, the amount of cellular RNA will change with growth rate in continuous culture or with growth phases of batch cultures. The ratio of RNA to DNA can therefore be used to measure metabolic activity (growth rate, μ). Amounts of RNA and DNA can be quantified by a number of methods. However, most methods such as tritiated thymidine or uridine incorporation into DNA or RNA (12) or orcinol and diphenylamine assays for RNA and DNA, respectively, are not specific for species, but measure the nucleic acid concentrations of the community as a whole. Therefore those methods cannot detect one species rapidly dividing while another species is approaching cell death. It would also be useful to determine whether an apparently stable population is metabolically active and to estimate the turnover of such a population. Variations in the metabolic activity between resin acid-degraders in

situ would have direct implications for the degradation of different resin acids under varying environmental conditions. As it was shown (20), the ability to degrade different resin acids is widespread among different subclasses of Proteobacteria, but the individual bacterial isolates can be very substrate-specific. Therefore, in a diverse activated sludge community it is advantageous to measure the metabolic activity of particular species which can degrade a type of resin acid under certain environmental conditions.

Hypervariable regions of ribosomal RNA molecules (rRNA) and their genes (rDNA) are ideal targets for species-specific gene probes (5) (32). 16S rRNA or 16S rDNA probing have found wide applications in in situ hybridization (1) (29), slot blot hybridization (23) (35), and PCR (7). Quantitative species-specific slot blot hybridization augments existing methodologies, such as in situ hybridization, which is commonly used to describe the spatial distribution of species within their natural environments. Attempts have been made to use in situ hybridization with fluorescently tagged species-specific oligonucleotide probes to quantify the metabolic activity of single cells within biofilms (24) (26). Others (31) have combined structural analysis of nitrifying biofilms using in situ hybridization with functional analysis using microelectrodes. The application of slot blot hybridization to measure metabolic activity of desired species may overcome limitations of existing hybridization techniques: it is not hampered by the penetration of hybridization probes through cell membranes, it can analyse nucleic acid samples representative of large numbers of cells, and does not require additional measurments.

We previously found that the RNA:DNA ratio of a number of resin acid degrading bacteria is positively and linearly correlated with growth rate, but the slope of the functions relating RNA:DNA ratio to growth rate varied between different strains (21). This variation emphasizes the need for calibration when the RNA:DNA ratio is used to measure growth rate of different bacterial isolates. Previous publications also show both, exponential (2), (18) as well as linear (11) relationships between growth rate and RNA per cell. A summary plot of RNA:DNA ratios (14) reveals a linear correlation to growth rate in different species. We previously used slot-blot hybridization with oligonucleotide probes to trace the changes in RNA:DNA ratio in an axenic batch culture of the resin acid degrading isolate Sphingomonas sp. DhA-33. The RNA:DNA ratio peaked during exponential growth phase and declined rapidly during stationary phase. This result confirmed similar observations made by Wade and Morgan (11).

Recently, Cangelosi et al. (3) have suggested the use of precursor-rRNA as an indicator for microbial activity, rather than mature rRNA, due to the rapid breakdown of the precursors upon cessation of growth. While the response of the pre-cursors may indeed be faster than of mature RNA, we observed similarly rapid changes in the RNA:DNA ratio in resin acid-degraders (21) (11). Also, fluctuations in the pre-rRNA pool seem to vary quantitatively with the conditions limiting growth (3) . The RNA:DNA ratio was shown to vary solely with growth rate, and was independent of the substrate used (2). Moreover, in some environments, precursor-16 rRNA processing may be inhibited (17), making this molecule difficult to use for growth rate estimates.

The objectives of this study were to a) determine the relationship between specific growth rate and 16S rRNA:rDNA ratio during steady-state growth of DhA-33, b) determine the population and metabolic dynamics of DhA-33 when it has to compete for nutrients with other sludge microorganisms in batch culture, c) to determine the efficiency of resin acid (dehydroabietic acid) removal of the sludge amended DhA-33, and d) to test the applicability of the species-specific hybridization method in a simulated wastewater treatment system. In this paper, we demonstrate that quantitative slot-blot hybridization can measure changes in the

RNA:DNA ratio of one strain, DhA-33, when that strain grows in competition with other activated sludge bacteria.

MATERIALS AND METHODS

Bacterial strain. The resin acid-degrading microorganism Sphingomonas sp. DhA-33 used in this study was previously isolated from an experimental sequencing batch reactor (SBR) (19). The identity of DhA-33 is based on polyphasic classification (20) , and DhA-33 was found to be most closely related to Sphingomonas yanoikuyae (ATCC 51230).

Batch culture on BR medium. 250 mL BR medium (19) with 1 g/L arabinose as carbon source was inoculated (1.0%) with an exponential growth phase culture of DhA-33. The culture was grown at 30 ^{o}C at a shaker speed of 250 rpm so that oxygen was non-limiting. The sample volume was adjusted, depending on the cell density at the time of sampling (25-50 mL for low biomass and 5-10 mL for high biomass).

Chemostat cultures. A 3-L applicon vessel (Applicon Dependable Instruments, Holland) was used to maintain a 2-L pure culture of DhA-33 at steady-state over a range of growth rates. The growth medium used was a mineral medium (BR) plus the wood sugar arabinose (1 g/L). The medium was kept in a 20-L reservoir at room temperature. The reactor temperature was maintained at 30 ^{o}C; the pH, at 7.0 using ammonium hydroxide; and dissolved oxygen, at 70% using a BioController ADI 1030 and Motor Controller ADI 1012 from Applicon. The reactor was inoculated (1.0%) with an exponential-phase batch culture of DhA-33 and run in batch mode. When the OD approached 0.5, the batch reactor was switched over to chemostat mode. The inflow of fresh medium and the outflow of spent medium and cells were regulated by a Minipuls 3 peristaltic pump (Gilson, Villiers, France). The flow rates were measured at the outflow in triplicate using a 10-mL graduated cylinder, and steady-state growth rates were subsequently calculated to be 0.040, 0.062, 0.133 and 0.170 h^{-1} (μ = F/V at steady-state, where F is flow rate and V is culture volume). Triplicate samples for nucleic acid (NA) analysis (100 mL total) were taken from a sample port approximately two volume changes after the culture had reached steady-state, as determined by OD_{610}. Antifoam (Sigma, St. Louis, USA) had to be added periodically.

Axenic and mixed batch cultures on BKME medium. DhA-33 and activated sludge (kindly provided by Harmack Pacific Inc.) were grown on bleached kraft mill effluent (BKME) (Western Pulp Ltd., Squamish) and BR (1:1 v/v) plus dehydroabietic acid (100 ppm). Both, DhA-33 and sludge inocula were acclimatized by two passages on the medium prior to the experiment. The inocula (0.5 %) were taken from exponential phase cultures of DhA-33 or sludge of similar OD. For the experiment, DhA-33 and sludge were each grown separately, as controls, or together, as a consortium. Each treatment was carried out in triplicate in a volume of 250 mL. Flasks were incubated at 30 ^{o}C at 250 rpm. Samples were withdrawn to measure OD_{610}, dehydroabietic acid concentration, and NA concentrations.

Resin acid analysis. Dehydroabietic acid (DhA), with 12,14-dichlorodehydroabietic acid as an internal standard, was quantified by extraction with ethyl acetate, derivitization with diazomethane, and analysis by gas chromatography as previously described (19).

Nucleic acid extractions. Cells were harvested in DEPC-treated oakridge tubes by centrifugation at 12 000 x *g* for 10 min at 4 ^{o}C. The cells were washed once in DEPC-treated sterile saline solution, and cell pellets were frozen at -70 ^{o}C until NA extraction. RNA and DNA for hybridization experiments were extracted simultaneously in triplicate. Samples of batch cultures and chemostat cultures on BR medium, were extracted according to Giovanni (10) by

SDS/proteinase K digestion (10 min, 65 ^{o}C) and purified using phenol (saturated with DEPC-treated water) and chloroform-isoamylalcohol (24:1 v/v). Samples of activated sludge and DhA-33 grown on BKME were extracted by short SDS/proteinase K digestion (3 min, 65 ^{o}C) and hot phenol extraction, followed by homogenization of the interface and organic phase (300 µL of organic phase, 200 µL TE buffer, 250 µL glass beads) two times for 2.5 and 1 min at 5000 rpm using a beadbeater (Biospec Products Inc.). Aqueous phases from the phenol extraction and the two homogenizations were pooled and extracted with chloroform-isoamyl alcohol twice. All NA samples were precipitated with ethanol, dissolved in TE and divided into two aliquots. Each aliquot was digested with either RNase A or DNase I according to manufacturers' specifications (Pharmacia Biotech). DNA and RNA samples were stored at -70 ^{o}C. All glassware and solutions used were treated with DEPC (ICN Biomedicals Inc.) to eliminate external RNase contamination.

Slot blot hybridization and probe design. Each of the triplicate NA extracts was then slot blotted onto nylon membranes in three ten-fold dilutions to ensure that the target NA were in the linear range of the standard curve. A Schleicher & Schuell minifold II apparatus was used. DNA was boiled for 5 min and diluted in buffer to a final concentration of 10 x SSC. RNA was resuspended in RNA blotting buffer (formamide : formaldehyde : 10 x MOPS, 5:1.62:1 v/v), incubated for 15 min at 65 ^{o}C, and diluted to a final concentration of 10 x SSC. Probes used were designed to hybridize strain-specifically to DhA-33. Probe S-S-DhA33-70-a-A-20 (sequence: 5' CGC CAC TAC CAC CGA AGT GA) (Oligonucleotide probe database nomenclature) hybridized to 16S rRNA. Probe S-S-DhA33-107-a-S-20 (sequence: 5' TCA CTT CGG TGG TAG TGG CG), which is the reverse complement of the first probe, hybridized to rDNA. The probes' species-specificity was checked using the Ribosomal Database Project, the 16S rRNA database of the Technical University Munich, and experimentally by hybridization to a number of organisms likely to be encountered in wastewater treatment systems as well as to the closest relatives of DhA-33, Sphingomonas yanoikuyae (ATCC 51230) and Sphingomonas chlorophenolica (ATCC 33790). The probes were 5'endlabeled with (γ-^{32}P) ATP (ICN Radiochemicals) using T_4 polynucleotide kinase (Gibco BRL). Overnight hybridization of DNA was performed at 35 ^{o}C, and of RNA at 45 ^{o}C. Stringent wash temperatures were found to be 47.5 ^{o}C for DNA and 52.5 ^{o}C for RNA. A PhosphorImager and ImageQuant (Molecular Dynamics, Inc.) were used to quantify hybridization signals. The signal intensity of the highest RNA concentration often saturated the phosphorimager; therefore, the next dilution was used for the ratio calculation. Signal intensities for the DNA were lower than for the RNA, and therefore the highest DNA concentration was used for calculations. Differences in dilutions or sample volumes were accounted for in all ratio calculations. Standard curves, relating spectrophotometrically measured mass amounts of RNA and DNA to hybridization signals, were produced. To determine the detection limit, the hybridization assay was done with known amounts of DhA-33 cells mixed with E. coli cultures (cell densities of 10^7 to 10^9 per ml) and mixed with activated sludge (cell density of approximately 10^9 per mL).

Statistics: The variance of a ratio was calculated using the following relationship: $VAR\ (Y/X) = Y^2/X^2 \times [\ (VAR(Y) / Y^2) + (VAR(X) / X^2) - (2 \times COV(X,Y) / (X \times Y)\ ]$, (where Y corresponds to the mean of triplicate samples of RNA and X is the mean of triplicate samples of DNA).

RESULTS AND DISCUSSION

Correlation between RNA:DNA ratio and μ. Using a chemostat, we were able to correlate the specific growth rate of DhA-33 to its 16S rRNA:rDNA ratio. During equilibrium growth, the RNA:DNA ratio increased linearly with increasing growth rates (Fig. 1). This positive linear correlation between growth rate and RNA:DNA ratio agrees well with previous studies on other resin acid degrading isolates, on a sulfate reducing bioreactor isolate PT2 (26), P. stutzeri Zobell (14), E. coli (2), S. typhimurium (30), and A. aerogenes (22). The slow-growing marine isolates in the study by Kemp et al. (13) showed a positive linear correlation between growth rates and RNA:DNA ratio when plotted on a semi-log plot. It seems that fast growing strains, such as E.coli, and slower-growing environmental isolates have a similar relationship between RNA:DNA ratio and growth rate. The variety of organisms studied, although small in number, indicates an underlying common mechanism for the regulation of the RNA:DNA ratio and growth rate. This relationship can be exploited for measuring the growth rate of bacterial species in pure cultures and complex communities.

The relationship between amount of RNA or DNA per slot and radioactive signal intensity was not linear but rather logarithmic as evident from standard curves (Fig. 2. At low RNA concentrations (0-650 μg), the relationship between RNA and signal intensity was linear, however, at higher concentrations, the signal intensity approached a maximum. DNA signal intensities had a logarithmic function even at low DNA concentrations. It was therefore desirable to slot-blot several dilutions of RNA and DNA samples and to measure the signal intensity in the linear or lower range of the standard curves. When aliquots of the same samples were extracted and analyzed a second time, the correlation between RNA:DNA ratio and growth rate was confirmed (data not shown). However, the slope, based on signal intensities, increased by a factor of ten. This variation can be attributed to a) experimental variability; b) different probe labeling efficiencies of the RNA and DNA probes in both experiments; and c) the dilutions of RNA and DNA on the membrane used for the calculation of the ratio. The differences between experiments exemplify the requirement for a standard curve for each experiment, to allow comparisons between experiments. However, datapoints of experiments conducted with the same batch of radiolabelled probes can be compared to each other, because the specific radioactivity of the oligonucleotide probes will not change, and triplicate application of each sample to the membrane will account for variations in binding efficiencies across the membrane (27).

RNA:DNA ratio during batch culture on BR medium. The RNA:DNA ratio of DhA-33 increases during early exponential growth phase, has a maximum at mid- to late-exponential growth phase, and then decreases during extended stationary phase to below the original levels (Fig. 3). These trends are consistent with earlier findings (21) and unpublished data. The maximum ratio here is slightly lower than previously reported, but depends on probe labeling and hybridization efficiencies as well as on the dilutions used for calculations, as described above. In addition, we have plotted the amounts of DNA and RNA in μg/mL, calculated using a standard curve of known amounts of DNA and RNA for hybridization experiments similar to the one in figure 2. The DNA exponentially increases during exponential growth until hour 16, reaches a maximum of 8 μg/ml at hour 22 and then remains constant, a trend very similar to the one seen for optical density. This indicates that the concentration of the DNA in a cell does not change substantially, especially in slower-growing strains such as DhA-33. The correlation coefficient of DNA versus optical density during exponential phase, was 0.9554. However, when adding stationary phase data, the overall correlation became weaker, R^2=0.7861. This may be due to the fact that during stationary phase cells become smaller, harbouring relatively more DNA per unit biomass (optical density). Alternatively, the cells' membranes may leak, reducing their OD, while

their DNA remains intact. These results indicate, that a species-specific measurment of DNA can be used as an approximation of the biomass of that species. The RNA increases at a higher rate than the DNA during logarithmic growth phase. The maximum RNA concentration of 28 μg/mL is at 16 hours, which is earlier than the maximum DNA concentration. This indicates that the RNA concentration per unit biomass decreases by the end of the exponential growth phase. During stationary phase, the RNA concentration per unit biomass decreases further.

The resin acid-degrading strain, DhA-33, was isolated from a sequencing batch reactor (SBR) which is very similar to a batch culture. We examined a complete growth curve of DhA-33 in order to apply the hybridization methodology in the SBR or other mixed batch cultures. DhA-33 increases its RNA:DNA ratio two-fold during logarithmic growth phase, and decreases the ratio in late stationary/death phase to below the original levels during lag phase. The ratio during lag phase is similar to the ratio during early stationary phase. This is interesting, as the batch culture was inoculated with cells growing at exponential phase, and indicates that the cells quickly reduced their RNA:DNA ratio when transferred to fresh medium. The RNA:DNA ratio in late stationary phase (t = 36 hours) was very low, indicating a severe reduction of RNA synthesis in strain DhA-33. Interestingly, inocula taken from DhA-33 at this phase did not grow under the same media and temperature conditions, indicating that either cell death had occurred (but not cell lysis as monitored microscopically) or that cells have entered a viable but non-culturable state. We did not attempt to examine this question further. Davis et al. (4) observed similar trends in phosphorus-starved E. coli cells, which lost viability along with degradation of ribosomes. However, Fukui et al. (9) showed that in a starved population of Desulfobacter latus, the mean ribosomal RNA content decreased only to 30% of the RNA content of growing cells and stayed constant during prolonged starvation. This observation, as well as the high survival rates of this strain, suggest that D. latus uses the K-strategy for survival: at very low nutrient levels, a relatively large amount of rRNA is maintained, so that sudden availability of substrate can be exploited. These different survival strategies have to be taken into account when the RNA:DNA ratio is used as an indicator of metabolic activity for various bacterial strains. It should be noted that the SBR from which DhA-33 was isolated was on a 24 hour cycle regime, which means that the cells had about 23 hours to complete a growth cycle. Strain DhA-33 is presumably adapted to the 24 hour cycle of the reactor. Interestingly, the 24 hour cycle of the SBR is similar to the time required for a batch culture on similar medium (see Fig 4A). At 24 hours, strain DhA-33 remains "culturable" and able to grow as soon as new wastewater is added and aeration restarts.

This study also shows that interpretation of the RNA:DNA ratio has to involve consideration of the environmental conditions under which the samples were taken. When environmental conditions change, such as in a batch culture where nutrients are depleated over time, the RNA:DNA ratio will change as well. The hypothesis that the RNA:DNA ratio is directly proportional to growth rate was developed on the basis of cell cultures under steady-state conditions, but is now applied to environmental scenarios, which are most likely not at steady-state. The RNA:DNA ratio tends to be more complex under non-steady state conditions, as shown in the batch culture, where the ratio increased during exponential phase (Fig. 3). As we encounter variations in the RNA:DNA ratio of a species from environmental samples, there may be two different possible explanations: The ratio is related to the specific growth rate of a bacterium growing exponentially under steady-state conditions or, the ratio is responding to different growth stages under non-steady-state conditions. An example would be a low RNA:DNA ratio, which could indicate a species well adapted to oligotrophic conditions growing

very slowly, but exponentially, or which could indicate a species not adapted to oligotrophic conditions in lag or stationary phase. Therefore, in addition to considering environmental conditions, it is also useful to study the organism of interest in pure culture to know how much the RNA:DNA ratio varies with growth rate. It should be possible to directly measure growth rate in continuous wastewater treatment systems by quantifying the RNA:DNA ratio. However, in non-steady state treatment systems, the RNA:DNA ratio would be a more general estimation of growth. This estimation would be dependent upon other measurments of environmental conditions and prior knowledge about the organism of interest.

Competition experiments on BKME medium. Mixed cultures were inoculated with activated sludge, obtained from an activated sludge secondary treatment system at a pulp and paper mill, plus strain DhA-33. Both, sludge and DhA-33 inocula were of similar OD and growing exponentially at the time of transfer. 16S rRNA hybridization did not detect DhA-33 in the activated sludge. However, due to the detection limit, DhA-33 could have occured in the sludge at about 10^6 cells per mL. The resin acid DhA was completely removed from the BKME/BR medium during growth of all three treatments: sludge, DhA-33, and sludge plus DhA-33 (figure 4B). DhA was degraded at the same rate in cultures of DhA-33 or of sludge alone. When DhA-33 and sludge were grown as a co-culture, DhA was degraded more rapidly. Complete removal of DhA was achieved about 10 hours earlier in the co-culture than in cultures containing DhA-33 or sludge alone. The positive influence of DhA-33 on the efficiency of DhA removal in the co-culture suggests that DhA-33 may be of further interest for studies of re-inoculation of treatment systems inactivated by stresses. It should be noted that all three treatments received the same aeration and maintained a pH of 7.0 throughout the incubation, thereby eliminating factors that could have caused differential DhA removal. Measurement of OD_{610} was not sufficient to accurately evaluate growth of the three different treatments because of interference due to the formation of sludge flocs, precipitation of DhA and the high turbidity of the BKME. OD increased in all treatments indicating growth of the sludge and of DhA-33. OD of DhA-33 alone increased by a factor of 1.3, while OD of sludge alone and of sludge plus DhA-33 increased by a factor of 1.7 suggesting that all three treatments grew successfully and that the sludge contributed more to the overall growth of the mixed culture. Endogenous respiration was evident by a decrease in OD in all three treatments after 35 hours. Measurement of OD in triplicates varied widely (data not shown).

Quantitation of the 16S rDNA of DhA-33 revealed that the strain grew during the first 24 hours in both treatments containing DhA-33 (figure 4A). The growth rate was similar for both treatments. The yield of DhA-33 in axenic culture was twice that in the mixed culture. This confirms that sludge cells contributed more to the overall growth in mixed cultures as already evident by optical densities. DNA samples of DhA-33 in activated sludge were corrected for background hybridization signals using non-specific hybridization signals from activated sludge samples from the same timepoints. Non-specific hybridization to sludge DNA increased with growth, but signal intensities were 50% less than signal intensities from species-specific hybridization, especially during maximum DhA-33 cell densities. The specificity of the hybridization probes to DhA-33 was confirmed as described in Materials and Methods. It appears that DNA hybridization has a higher background signal than RNA hybridization, perhaps due to the higher sequence variability in DNA.

The maximum and minimum RNA:DNA ratios of DhA-33 were not significantly different for the two treatments (Figure 4C). When DhA-33 was grown without competition, the RNA:DNA ratio increased sharply to 1.6 during logarithmic growth phase and then decreased

when the cells entered stationary phase to original levels. This result is very similar to the result obtained on BR medium amended with arabinose. When DhA-33 was grown in competition with other sludge bacteria, the initial RNA:DNA ratio was higher and decreased sooner than in cultures without other sludge organisms. At stationary phase the RNA:DNA ratio decreased to below initial levels.

Strain DhA-33 grew and maintained itself in batch co-cultures with activated sludge and was not out-competed by other organisms. This was shown by quantitation of DhA-33 rDNA (Fig. 4A). Species-specific DNA hybridization can therefore be a useful assay in environments where particulates as well as other organisms interfere with more conventional measurment techniques such as optical density. In the co-cultures, DhA-33 presumably had to compete for nutrients with other sludge organisms. This competition is probably why the final DNA concentration and final cell density of DhA-33 were lower in the sludge co-cultures than in the axenic DhA-33 cultures. The rate of DhA-33 DNA increase was only slightly higher in axenic DhA-33 cultures without sludge. It therefore appears that DhA-33 growth rates were similar despite the competition from other organisms. This is in agreement with similar maximum RNA:DNA ratios in the two cultures. The RNA:DNA ratio increased during growth and decreased during stationary phase in both treatments, as expected from previous pure culture experiments. The RNA:DNA ratio of DhA-33 decreased earlier and faster in DhA-33/sludge co-cultures, presumably due to an earlier depletion of nutrients, especially of DhA as shown in figure 4A. This is also reflected by an earlier entry into stationary phase of DhA-33 in co-cultures.

Detection limits. We observed different detection limits for RNA and DNA, respectively. RNA from DhA-33 could be detected at a target cell density of 10^6, and DNA at a target cell density of 10^7. The difference in detection limit between RNA and DNA can be explained by the higher amount of ribosomal RNA in the cells compared to rDNA. The detection limit was negatively affected by the concentration of additional non-target E.coli cells (not shown).

There were no differences in the detection limit between nucleic acids extracted from a defined bacterial culture and nucleic acids extracted from activated sludge, indicating that the extraction procedure works well for floc-forming mixed sludge cultures containing impurities such as fibreous material and high molecular weight organic compounds. However, the overall detection limit of 10^7 target cells per mL for slot-blot hybridization limits this technique to organisms present in their environments at high cell densities. In dense activated sludge communities, a target organism must comprize about 1% of the total community.

Applicability of the method / Conclusion. We were able to show that quantitative and species-specific slot blot hybridization can be applied to measure the abundance and the metabolic activity of a species in an activated sludge system degrading dehydroabietic acid. The method is based on the relationship between RNA:DNA ratio and growth rate which is linear and positive in Sphingomonas sp. DhA-33. While hybridization to measure abundance is not new, the combination of hybridization to DNA and RNA and the application of the RNA:DNA ratio as a species-specific measure of metabolic activity in an activated sludge system is. Quantitative hybridization can be used to accurately monitor a strain of interest in simulated waste water treatment systems to further elucidate it's role in the resin acid degrading activated sludge community. Application of this method may include determining the reaction of a species to stresses (e.g., black liquor spills or alkaline spills in pulp and paper mills), to an increase of temperature in the recycling water of closed-loop mills, to a decrease of temperature in lagoons or activated sludge systems in the winter months and to shut-down and restart of a mill and it's treatment system. Of interest may be, whether these stresses kill or just inactivate populations.

Where populations sizes are stable, the turnover rate of the population could be estimated from its growth rate.

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FIGURE LEGENDS:

FIG. 1. Linear relationship between growth rate (μ) and 16S rRNA:rDNA ratio of DhA-33 at equilibrium growth in a chemostat. (n=3; error bars indicate variance of the ratio)

FIG. 2. Standard curve relating phosphor imager signal intensities (PI) to mass amounts of total cellular RNA and DNA.

FIG. 3. Trends of DNA (■), RNA (▲), and RNA:DNA ratio (●) during batch growth of DhA-33 (◆) on BR mineral medium and arabinose.

FIG. 4. (A) Growth of DhA-33 on BKME plus DhA as determined by DNA hybridization (n=3; error bars indicate standard variation), (B) degradation of DhA (n=3; error bars indicate standard variation), (C) RNA:DNA ratio of DhA-33 growing axenically and in competition with activated sludge (n=3; error bars indicate variance of the ratio, ratio is based on conversion of phosphorimager signal to ng/ml of NA).

FIGURES

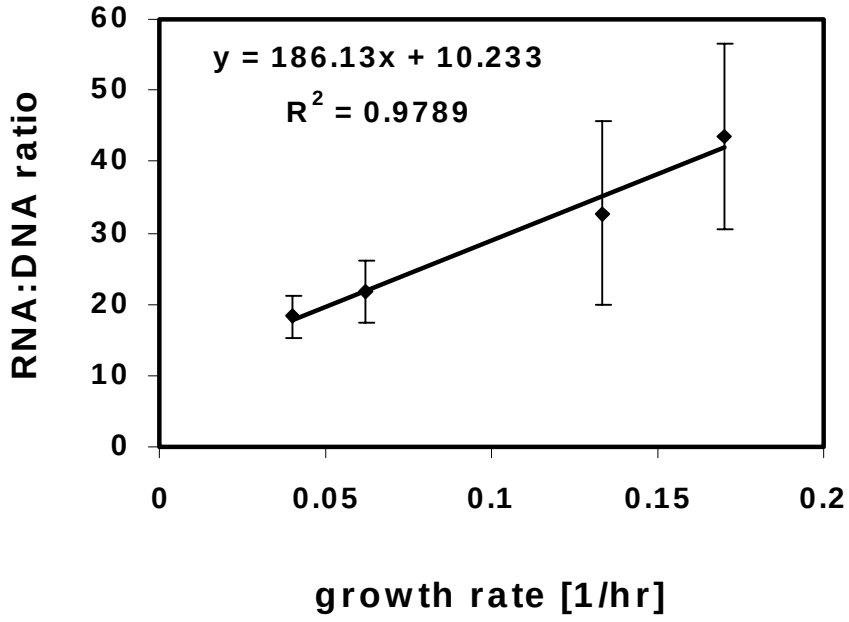

Figure 1.

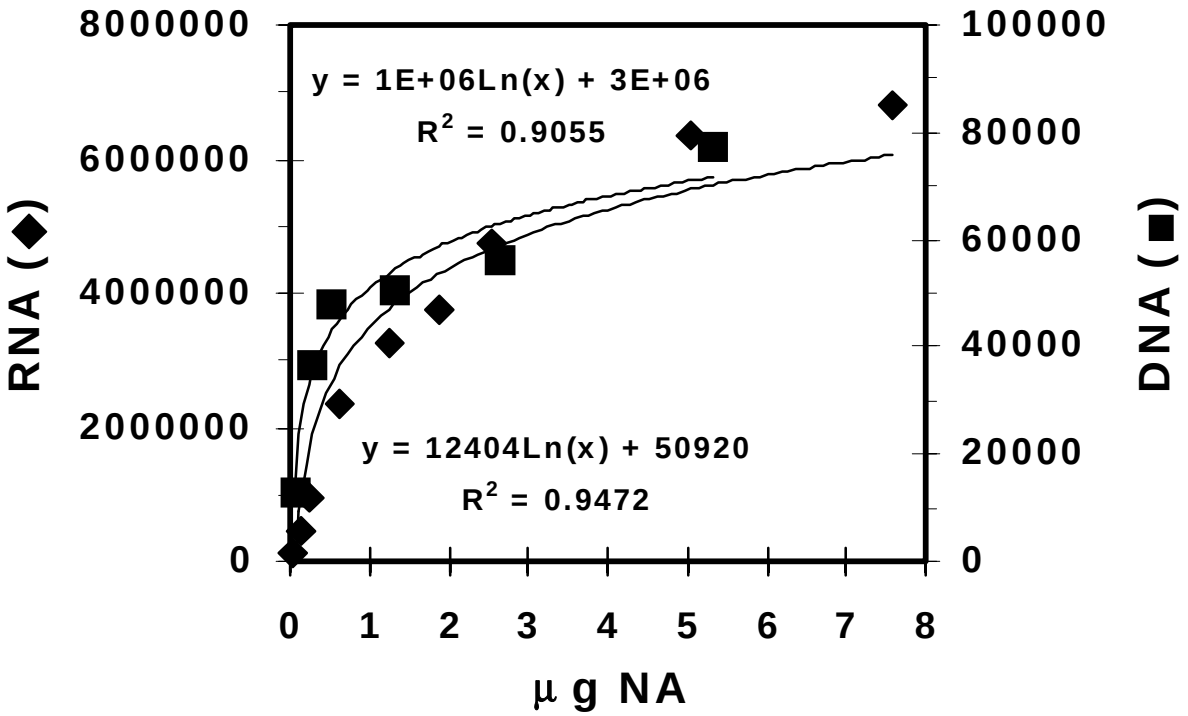
8000000
6000000
4000000
2000000
0
RNA (◆)
100000
80000
60000
40000
20000
0
DNA (■)
y = 1E+06Ln(x) + 3E+06
R2 = 0.9055
y = 12404Ln(x) + 50920
R2 = 0.9472
0 1 2 3 4 5 6 7 8
µg NA

Figure 2.

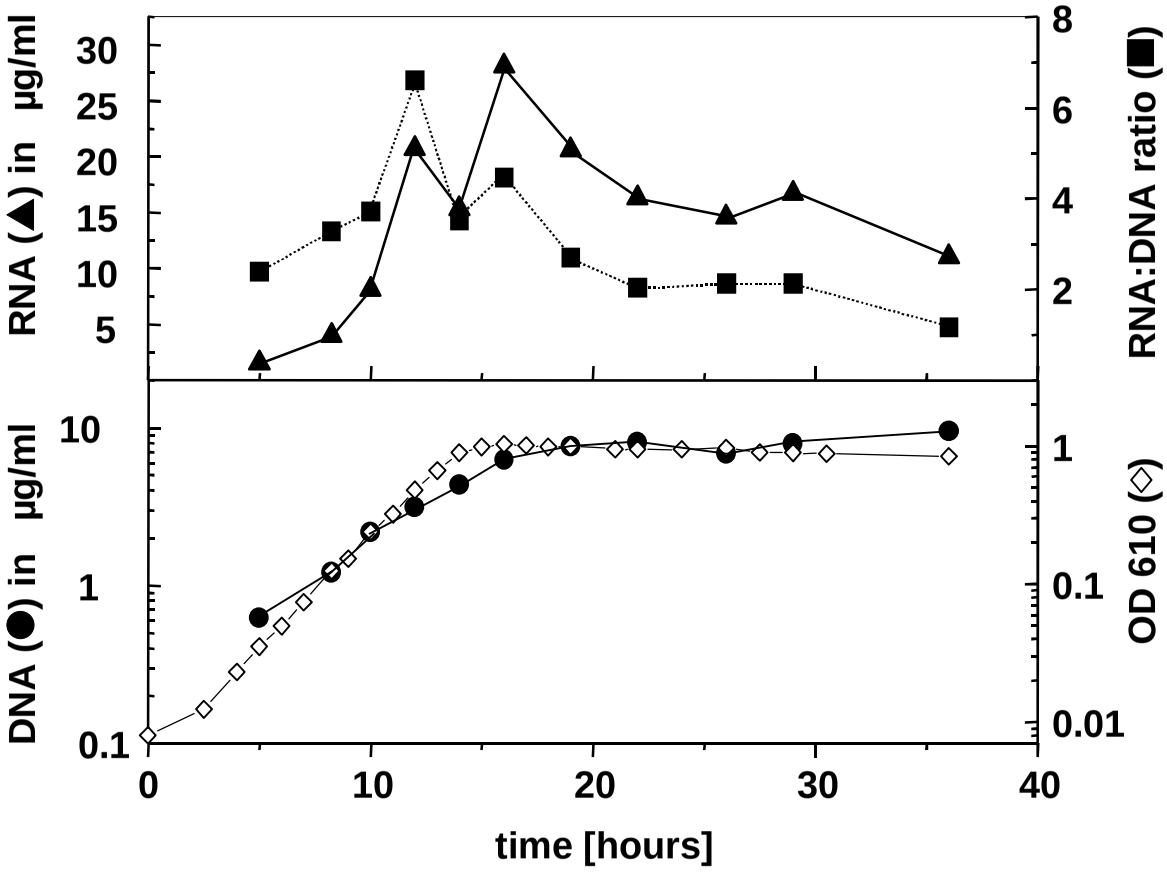
RNA (▲) in µg/ml
30
25
20
15
10
5
8
6
4
2
RNA:DNA ratio (■)
DNA (●) in µg/ml
10
1
0.1
1
0.1
0.01
OD 610 (◇)
0
10
20
30
40
time [hours]

Figure 3.

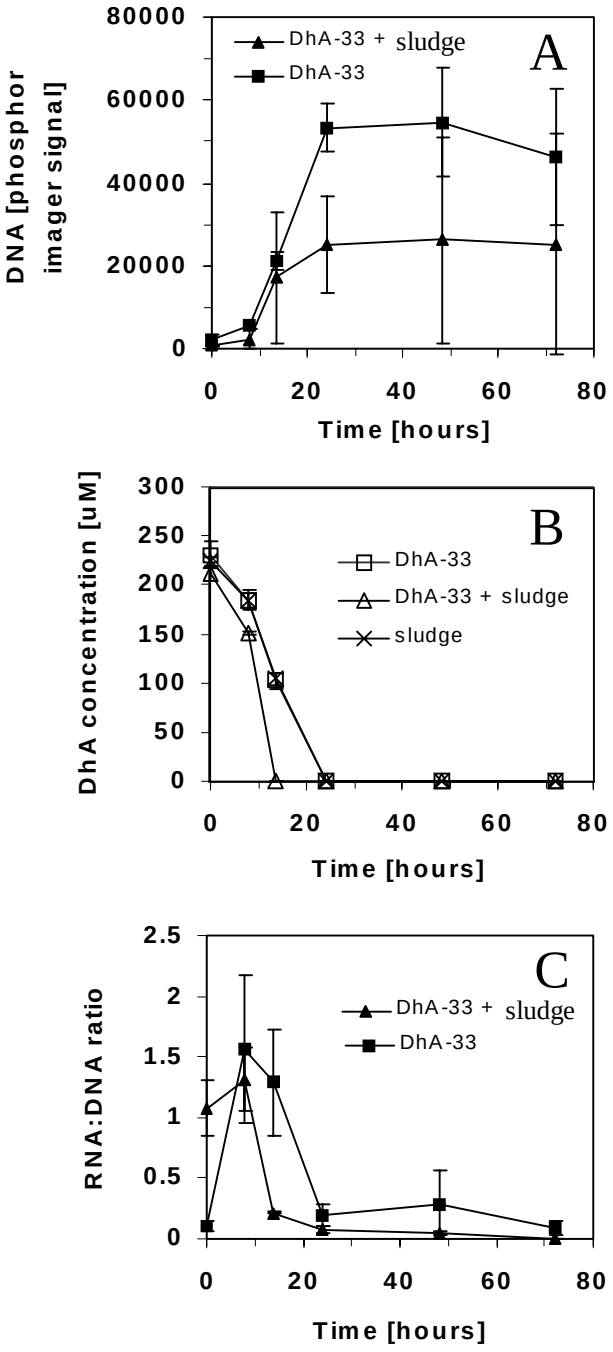
A
DhA-33 + sludge
DhA-33
DNA [phosphor imager signal]
80000
60000
40000
20000
0
0
20
40
60
80
Time [hours]
B
DhA-33
DhA-33 + sludge
sludge
DhA concentration [uM]
300
250
200
150
100
50
0
0
20
40
60
80
Time [hours]
C
DhA-33 + sludge
DhA-33
RNA:DNA ratio
2.5
2
1.5
1
0.5
0
0
20
40
60
80
Time [hours]

Figure 4.