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Detection of Gene Expression in Genetically Engineered Microorganisms and Natural Phytoplankton Populations in the Marine Environment by mRNA Analysis

SCOTT L. PICHARD AND JOHN H. PAUL*

Department of Marine Science, University of South Florida, 140 7th Avenue South, St. Petersburg, Florida 33701

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A simple method that combines guanidinium isothiocyanate RNA extraction and probing with antisense and sense RNA probes is described for analysis of microbial gene expression in planktonic populations. Probing of RNA sample extracts with sense-strand RNA probes was used as a control for nonspecific hybridization or contamination of mRNA with target DNA. This method enabled detection of expression of a plasmid-encoded neomycin phosphotransferase gene (*nptII*) in as few as 10^4 *Vibrio* cells per ml in 100 ml of seawater. We have used this method to detect expression of the ribulose-1,5-bisphosphate carboxylase large-subunit gene (*rbcL*) in *Synechococcus* cultures and natural phytoplankton populations in the Dry Tortugas, Florida. During a 36-h diel study, *rbcL* expression of the indigenous phytoplankton was greatest in the day, least at night (1100, 0300, and 0100 h), and variable at dawn or dusk (0700 and 1900 h). These results are the first report of gene expression in natural populations by mRNA isolation and probing. This methodology should be useful for the study of gene expression in microorganisms released into the environment for agricultural or bioremediation purposes and indigenous populations containing highly conserved target gene sequences.

The functioning of microorganisms in the environment is a direct result of their gene-encoded enzyme activities. Accurate methods of measuring the activities of enzymes that catalyze important ecological and geochemical processes have been sought. Our laboratory has become interested in the factors which regulate genes in environmental microbial populations. Our approach to this problem has been to study transcriptional regulation by using mRNA isolation and analysis.

The isolation of mRNA as a method for studying transcriptional regulation has been used in prokaryotic and eukaryotic cells for some time (11, 15). This approach is only beginning to be used in the field of microbial ecology (7a, 29).

An inherent problem in detecting microbial gene expression in a heterogenous bacterial population by mRNA analysis is the need for homology between target gene sequences and available probes. Thus, this approach is limited to the detection of genes of known organisms added to or released into the environment (exogenous genes) and highly conserved genes of indigenous flora (endogenous genes). We have chosen to study a plasmid-encoded antibiotic resistance gene (*nptII* [1]) as an exogenous gene and the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO) (*rbcL* [27]) as an endogenous gene. RUBISCO, the major carboxylating enzyme in photosynthesis, has been described as the most abundant protein on earth (7), and the nucleotide sequence for the *rbcL* gene is highly conserved (25). We have combined the method of Chomczynski and Sacchi (4) for RNA isolation with sense (5) and antisense (AS) probing to detect expression of both these genes in seawater.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study appear in Table 1. Our model exogenous gene (or gene that we thought would have a low probability of occurrence in aquatic environments) was the neomycin phosphotransferase gene (*nptII*) of Tn*5* which codes for kanamycin and neomycin resistance (14). This gene was on the broad-host-range IncP4 plasmid pQSR50, a derivative of R1162 (16). pQSR50 was maintained in *Escherichia coli* RM 1259 and in the marine *Vibrio* sp. strain WJT-1C(pQSR50) (8) (Fig. 1). *nptII* was directionally cloned into pGEM4Z by *Hin*dIII-*Bam*HI digestion of pQSR50 and standard cloning methods (15) (Fig. 1).

The *rbcL* used as the endogenous gene was originally from *Synechococcus* sp. strain PCC 6301 (formerly *Anacystis nidulans* 6301 [24, 25]). *rbcL* was subcloned in pGEM3Z by *Pst*I-*Eco*RI digestion of pCS751 (28) and standard cloning methods (15) (Fig. 1). For preparation of the purified *rbcL* gene for dot blotting, the restricted fragment was purified by low-melting-point agarose gel electrophoresis and Elutip-d (Schleicher and Schuell, Keene, N.H.) chromatography.

Culture conditions. *E. coli* RM 1259 was grown on LB broth at 37°C in the presence of 50 μg of kanamycin per ml and 25 μg of streptomycin per ml. *Vibrio* sp. strain WJT-1C(pQSR50) was grown at 22 to 24°C on ASWJP plus PY (18) containing 500 μg of kanamycin per ml and 1,000 μg of streptomycin per ml. *Synechococcus* sp. strain PCC 6301 was cultured in BG-11 medium (5) at 2,000 to 3,000 lx and 22 to 25°C.

Field sites and microcosm studies. For detection of gene expression in *E. coli* RM 1259 or *Vibrio* sp. strain WJT-1C(pQSR50) in seawater, unfiltered seawater was collected from Bayboro Harbor, St. Petersburg, Fla. For microcosm studies, logarithmically growing cells of RM 1259 or WJT-1C(pQSR50) were either collected by filtration on muffled (i.e., heated at 450°C for 4 h) GF/F filters or added to 100 ml of unfiltered seawater (Bayboro Harbor) and then collected

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
E. coli RM 1259	Δ*trpE5*(pQSR50)	Richard Meyer, University of Texas, Austin
Vibrio sp. strain WJT-1C(pQSR50)	*nptII*	8
Synechococcus sp. strain PCC6301 ATCC 27144	*rbcS rbcL*	American Type Culture Collection
Plasmids		
pQSR50 (=R1162::Tn*5*)	IncP-4 Sm^r Km^r	16
pGEM3Z, pGEM4Z	Cb^r; riboprobe vectors containing multiple cloning sites flanked by Sp6 and T7 RNA polymerase promoters	Promega, Madison, Wis.
pNPTII	Km^r Cb^r; pGEM4Z derivative containing *nptII* of Tn*5* from pQSR50	8
pCS751	*rbcL* Cb^r	F. Robert Tabita, Ohio State University
pLC1	*rbcL* Cb^r; pGEM3Z derivative	

by filtration on muffled GF/F filters. The filters were immediately processed for RNA extraction as described below.

For detection of *rbcL* expression by indigenous phytoplankton populations, two liters of water taken near Loggerhead Key, Dry Tortugas, Fla. was filtered onto a 25-mm-diameter muffled GF/F filter, and the RNA was extracted as described below. Samples were also filtered onto GF/F filters for chlorophyll *a* analysis. Chlorophyll *a* was determined fluorometrically (9). Sampling was repeated approximately every 4 h for 36 h.

RNA extraction (i) GIPS method. Reagents were made in either sterile, disposable labware or glassware that had been

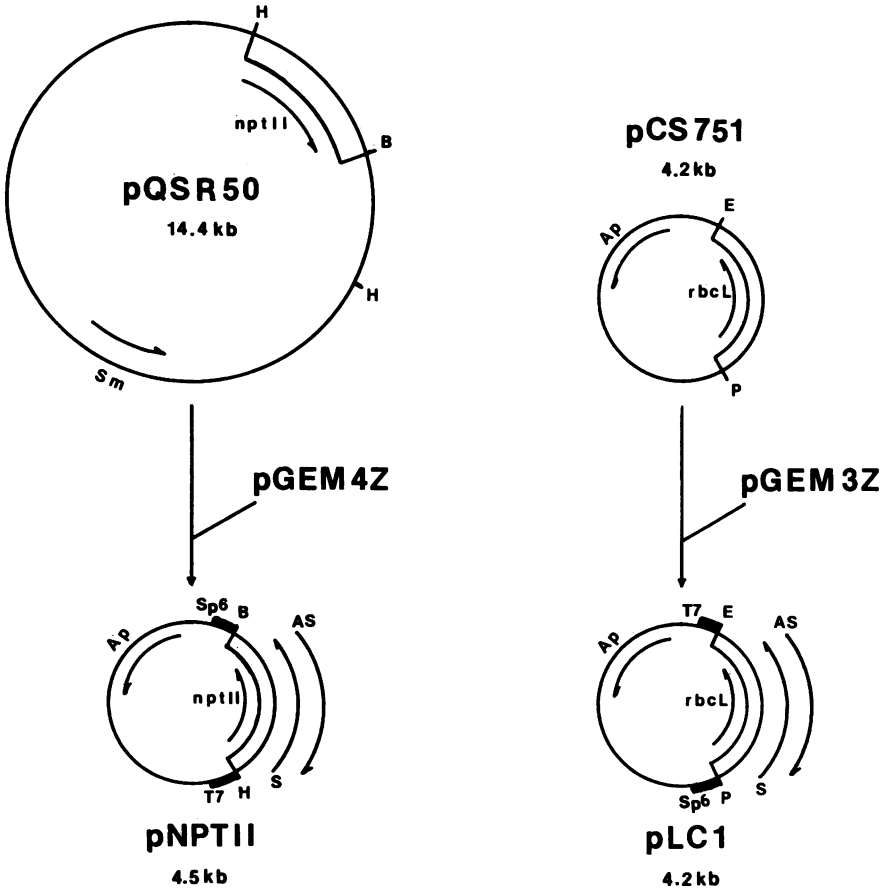

FIG. 1. Plasmids used in this study (see Table 1 for details). The constructs pNPTII and pLC1 were used to produce AS and S probes to the target genes *nptII* and *rbcL*, respectively. Abbreviations: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Sm, streptomycin; Ap, ampicillin.

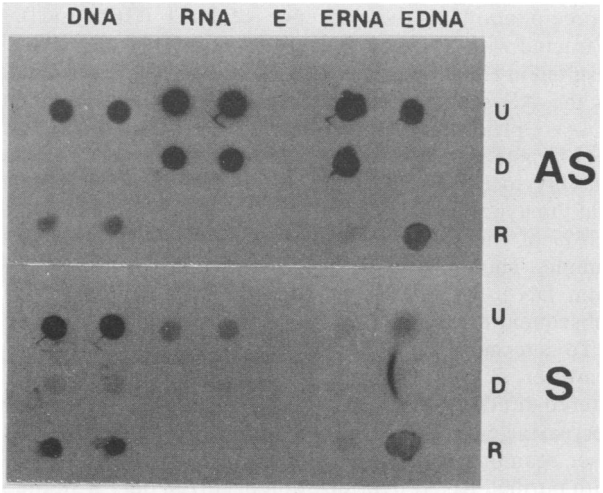

FIG. 2. Probing of purified *rbcL* DNA and RNA with AS and S RNA probes. The dots (5 ng each) in the row labeled U were undigested, whereas those in the rows labeled D and R were digested with DNase and RNase, respectively. An RNA extract from the water column is labeled E, and that extract spiked with *rbcL* RNA and DNA is labeled ERNA and EDNA, respectively.

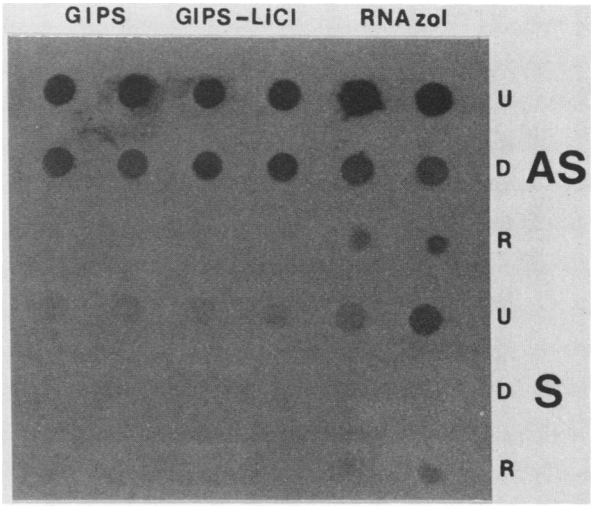

FIG. 3. Comparison of GIPS, GIPS-LiCl, and RNAzol extraction of *nptII* mRNA from *E. coli* RM 1259. The rows were labeled as in Fig. 2.

baked at 450°C for 4 h. All solutions were made with water treated with diethylpyrocarbonate (DEPC; Sigma Chemical Co., St. Louis, Mo.) (15). Cells collected either by filtration onto GF/F filters or harvested by centrifugation were extracted in 2.2-ml bead-beater microcentrifuge tubes (Biospec Products, Bartlesville, Okla.). For guanidinium isothiocyanate-phenol-sarcosyl (GIPS) extraction, 0.8 ml of a solution containing 4 M guanidinium isothiocyanate (International Biotechnologies, Inc., New Haven, Conn.), 0.5% sarcosyl, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 80 μl of 2 M sodium acetate (pH 4.0), 0.8 ml of water-saturated phenol, 160 μl of chloroform-isoamyl alcohol (49:1), and 8 scoops (~0.5 g) of glass beads (diameter, 0.1 to 0.15 mm; Biospec Products) was added to each tube. The tubes were placed in ice for 5 min followed by 1 min of bead beating using a microbead beater (Biospec Products). The tubes were cooled in ice for 15 min, followed by centrifugation at 10,000 × *g* in a microcentrifuge for 10 min. The aqueous layer was removed, and the filter was reextracted as described above. The aqueous layers were either precipitated with 1 volume of isopropanol at 2 h at −20°C or stored overnight (when necessary) at 4°C, followed by precipitation at −20°C. The RNA-containing pellets were harvested in a microcentrifuge for 10 min and dissolved in DEPC-treated water–1 mM EDTA, pH 7.0. The samples were precipitated with isopropanol as above and dissolved in DEPC-treated water–1 mM EDTA, pH 7.0. For the GIPS-LiCl method (19), after the first isopropanol precipitation, the pellet was resuspended in 0.1% DEPC-treated water–4 M LiCl by vortexing. The mixture was centrifuged at 4°C for 10 min at 10,000 × *g*, and the supernatant was discarded. The pellet was resuspended in 1 volume of a solution containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5% sodium dodecyl sulfate. After a further chloroform extraction, the aqueous phase was recovered, amended with 0.1 volume of 3 M sodium acetate (pH 5.0) and reprecipitated with isopropanol at −20°C for 1 h. The pellet was collected by centrifugation and resuspended in DEPC-treated water–1 mM EDTA.

(ii) RNAzol method. Cells collected on filters or cell pellets were extracted in 2.2-ml tubes by the addition of 1.0 ml of RNAzol (Cinna/Biotecx, Friendswood, Tex.), 0.1 ml of chloroform, and 8 scoops of glass beads. The remainder of the procedure was as for the GIPS procedure.

RNA dot blotting and probing. Each RNA extract was divided into three equal volumes; one volume was treated with an RNase-free DNase (RQ1 DNase; Promega), a second volume was digested with DNase-free RNase, and the third volume was untreated. For both nuclease digestions, $MgCl_2$ was added to a final concentration of 42 mM, and DNase digestion was in the presence of a placental ribonuclease inhibitor (final concentration, 4.7 U/μl RNasin; Promega). Nuclease digestions were for 30 min at room temperature. The DNase-treated and untreated samples were brought to a final volume of 500 μl with DEPC-treated water–1 mM EDTA, and 250-μl aliquots were dotted with a Bio-Rad BioDot dot blot apparatus for probing with AS and S probes. The RNase-treated sample was kept in a minimal volume (usually <30 μl) and dotted manually to prevent contamination of the dot blot apparatus. For production of RNA probes, the appropriate construct (pLC1 or pNPTII) was cut with a restriction enzyme distal to the RNA polymerase promoter and gene sequence of interest (Fig. 1). The linearized vector was then used to produce labeled transcripts. For example, cutting at the *Bam*HI site (labeled B in Fig. 1) of pNPTII and using the T7 RNA polymerase promoter resulted in production of a S transcript that was identical to the mRNA for *nptII*. By cutting this vector with *Hin*dIII and using the Sp6 polymerase promoter, an AS transcript (Fig. 1) would be produced that was complementary to the *nptII* mRNA. By using [^{35}S]UTP in the transcription reaction, ^{35}S-labeled RNA probes (AS or S) could be produced (8, 13). Hybridization and washing was performed at 55 and 65°C, respectively, as previously described (8). Certain dot blots were sprayed with an autoradiographic enhancer (En^{3}Hance; DuPont-New England Nuclear). Densitometry of X-ray films was performed with an LKB Ultroscan XL laser densitometer.

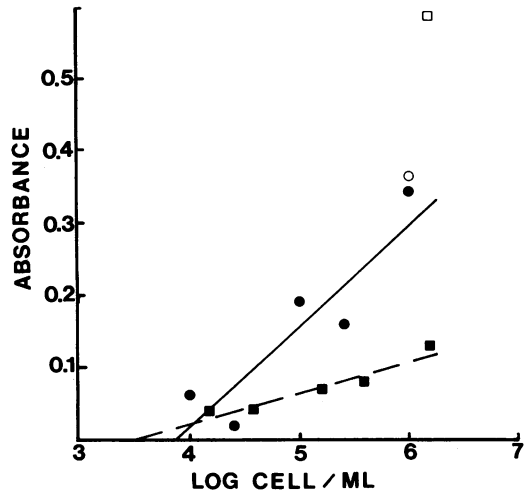

FIG. 4. Expression of *nptII* in *E. coli* RM 1259 (squares) or *Vibrio* sp. strain WJT-1C(pQSR50) (circles) as a function of cell concentration. Cells were either filtered directly from a culture (open symbols) or added at decreasing concentrations to 100 ml of seawater and filtered (solid symbols). The ordinate is the absorbance measured by laser densitometry of dot blots probed with the AS *nptII* gene probe.

RESULTS

Figure 2 shows the method for differentiation between purified *rbcL* RNA and DNA (5 ng each) by probing with AS and S transcripts. Hybridization to both the AS probe and S probe were found with purified DNA. RNase-treated samples hybridized to these probes, whereas DNase treatment resulted in no hybridization (Fig. 2). Purified RNA hybridized only with the AS probe and was digested by RNase. The slight hybridization with the S probe may have been caused by minute traces of the DNA template in our purified RNA transcripts. These data show that RNA can be easily differentiated from DNA by probing with AS and S RNA probes. Figure 2 also shows the results of probing of environmental RNA extracts of 25 ml of seawater spiked with purified *rbcL* RNA and DNA. Hybridization was identical to that obtained for RNA and DNA alone, indicating that nothing in the extracts interfered with the hybridization and detection processes.

Figure 3 shows the results of extraction of RNA from *E. coli* containing the *nptII* gene. Samples were either extracted by the GIPS method, extracted by the GIPS method with a reprecipitation in the presence of LiCl (GIPS-LiCl), or extracted with RNAzol. Extraction with GIPS or GIPS-LiCl resulted in isolation of mRNA, as indicated by hybridization to the AS probe and digestion by RNase. Although the RNAzol procedure isolated RNA, DNA was also present in the extracts, as indicated by hybridization to the S probe and stability to RNase digestion. Laser densitometry indicated that the hybridization intensity in the RNase-treated dot was ~16% of the untreated sample for the RNAzol-extracted sample. Thus, GIPS yielded a superior isolation of RNA from DNA than did RNAzol, and GIPS was used in all subsequent experiments.

To determine the capability to recover RNA from cells in seawater, *E. coli* or *Vibrio* cells containing *nptII* were either filtered directly from culture medium or added to seawater at decreasing cell concentrations. Figure 4 shows the results of laser scanning of an RNA dot blot of this experiment. As with purified RNA, hybridization occurred only with the AS probe and was removed by RNase digestion (data not shown). In general, a greater amount of hybridization was obtained with *Vibrio* cells added to seawater than with *E. coli* per equivalent number of cells. This is reflected in the slopes of the lines of regression of expression versus cell density (slope = 0.144 for *Vibrio* sp.; $r^2 = 0.83$; slope = 0.045 for *E. coli*, $r^2 = 0.92$). The hybridization obtained with RNA extracted from 1.5×10^8 *E. coli* cells was greater than that obtained for an equivalent number of cells suspended in seawater. Hybridization was detected at the lowest concentration examined, 1.5×10^4 cells per ml. Unlike the *E. coli* cells, equal hybridization was obtained from *Vibrio* cells filtered directly from culture or after resuspension in 100 ml of seawater. The detection limit was again about 10^6 cells total (10^4/ml of seawater; Fig. 4).

Figure 5 shows the results of studies on the extraction of *rbcL* mRNA from a *Synechococcus* culture and from natural phytoplankton populations from oligotrophic surface waters from the Dry Tortugas. Extraction of both the cyanobacterial culture and natural phytoplankton populations resulted in hybridization to the AS probe that was sensitive to RNase digestion, indicative of *rbcL* mRNA. In the natural population, the strongest hybridization occurred with samples taken during the day, with weakest hybridization for samples taken at night (i.e., 23, 03, and 01 h [Fig. 6]). The daylight/nighttime transitional samples (i.e., 19 and 07 h) had variable hybridization intensity. Also plotted in Fig. 6 is the chlorophyll *a* content at the time of sampling. In general, there was no diel trend in chlorophyll *a* content. These results indicate that changes in hybridization were more likely caused by

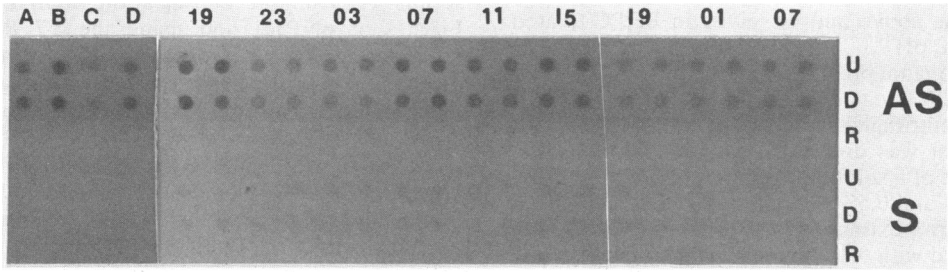

FIG. 5. *rbcL* expression in *Synechococcus* cultures (columns A to D) or natural phytoplankton populations sampled over 36 h in the Dry Tortugas (all other columns). Columns A and B contain extracts from cells (1 ml of culture of 1.2×10^8 cells) during a light phase of a light/dark growth cycle, and columns C and D contain extracts from cells during the dark phase of the growth cycles. All other columns correspond to the sampling time of day for the diel study (samples were taken in duplicate). The rows are labeled as in Fig. 2.

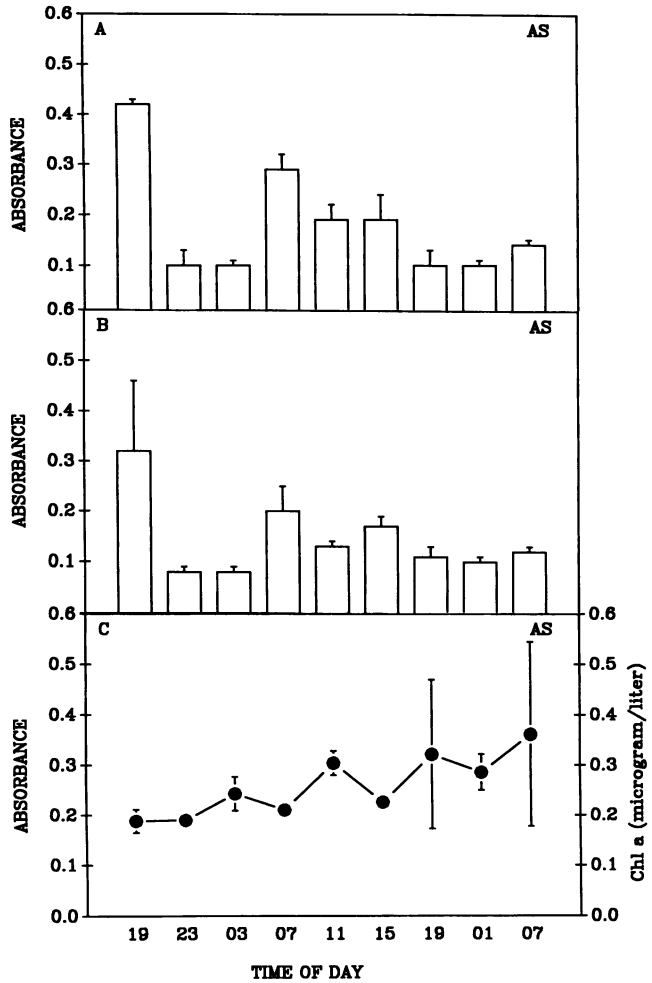

FIG. 6. Laser densitometry of the untreated row (A), the DNase-treated row (B), and the RNase-treated row (C) of the AS-probed dot blot in Fig. 5. Each band is the average value of the replicate samples for each time point. (C) Chlorophyll *a* (Chl a) concentration in the water at time of sampling.

differences in *rbcL* mRNA content than changes in size of the phytoplankton population.

DISCUSSION

We have combined the methods of Chomszynski and Sacchi (4) for guanidinium extraction of RNA with probing using AS and S RNA transcripts to detect gene expression in bacteria added to seawater and indigenous marine microbial flora. This methodology enabled detection of transcripts of a plasmid-encoded gene from as few as 10^4 cells per ml. Perhaps of greater significance, this procedure enabled detection of *rbcL* expression in phytoplankton from oligotrophic oceanic surface waters, representing the first report of detection of gene expression in the indigenous community by mRNA analysis.

The advantages of gene expression analysis based upon mRNA detection is that one basic technology (compared with multiple phenotypic or enzyme assays) is used to detect a range of microbially mediated processes, thereby simplifying the methodologies to be employed. Additionally, once total RNA has been extracted and blotted, the blots can be reprobed to detect expression of other genes.

mRNA isolation and analysis has not been widely used to understand the function of microorganisms in their native environments. Recently, Tsai and Olson (29) investigated regulation of expression of *merA* and *merB* in mercury-resistant bacterial isolates. ^{32}P-DNA probes were used to detect mRNA in slot-blotted guanidinium extracts of cells. The greatest level of *merA* mRNA was isolated from cells exposed to Hg^{2+} at 4°C (29). The large signal obtained at low temperature was attributed to the prolonged survival of mRNA in the cells at that temperature.

Guanidinium isothiocyanate has also been used to extract mRNA from *Synechococcus* sp. strain RF-1 to study the regulation of nitrogenase genes (10). Northern (RNA) blots were probed with ^{32}P-labeled *nifH* or *nifK* DNA probes, and diel periodicity in transcriptional regulation was observed. In cells grown with a light/dark illumination regime, nitrogenase mRNA was produced exclusively during dark cycles (10).

Fleming and Sayler (7a) isolated mRNA from *Pseudomonas putida* seeded in soil microcosms to detect expression of catabolic genes. RNA was extracted by bead beating in acetate, followed by phenol-chloroform fractionation and cesium chloride ultracentrifugation. DNase-resistant materials was found to bind to ^{32}P-labeled Nah A-D probes.

Our system for analysis of microbial gene expression is particularly suited for detection in environmental samples. A problem with studying genes in natural populations is the potential for nonspecific hybridization, caused by similar gene sequences of unknown function or impurities which might be present in RNA extracts. The S and AS probing protocols described herein provide a control for nonspecific hybridization in the same sample under identical hybridization conditions. Hybridization to the S probe will detect such nonspecific hybridization as well as contamination of the sample with target DNA.

The method described in this paper calls for five control treatments for each sample (two enzyme digests for AS probing and three samples for S probing). We believe the protocol could be further simplified by omission of the DNase treatment, thereby reducing the protocol to three controls. Contamination of the sample with DNA will be detected sufficiently by the S probing treatment. Additionally, we have occasionally found removal of all hybridizable material in the sample by DNase treatment, when no hybridization was found with the S probe. We believe this was caused by RNase in the sample that was not inhibited by the placental ribonuclease inhibitor. The placental ribonuclease inhibitor inhibits most but not all forms of RNase. Therefore, incubating RNA in the presence of Mg^{2+} as in the DNase assays may not be prudent for extracts from all types of samples.

The selection of GF/F filters for cell collection is a compromise between retention efficiency for loading capacity, fast filtration, and compatibility with extraction chemistry. Previous researchers have shown that 0.2-μm-pore-size membrane filters harvest bacteria more efficiently than glass-fiber filters. However, the filtration rate is too slow and the capacity is too small for analysis of ambient phytoplankton (or bacterioplankton) populations. Additionally, DEPC affects the integrity of both polycarbonate and cellulose nitrate filters.

There is considerable interest in the detection of microbial gene expression in the environment and particularly of indigenous microbial flora. Capone et al. (3) measured

expression of *nifH* genes using antisera raised against the amplified and cloned *nifH* gene maltose fusion products (31). Similarly, Orellana et al. (17) reported the use of anti-RUBISCO antibodies to detect this enzyme in phytoplankton cultures.

We chose to study RUBISCO expression because this enzyme is abundant in aquatic ecosystems and the primary structure of this enzyme has been somewhat conserved throughout evolution (27). The RUBISCO holoenzyme is a hexadecamer structure of equal numbers of small and larger subunits (L_8S_8 [27]). Although the nucleotide sequence of the small subunit gene is divergent among photosynthetic organisms, the large subunit is conserved, generally with 80 and 70% similarity at the amino acid and nucleotide levels, respectively, for cyanobacteria to higher plants (25). Chromophyte algae (chrysophytes and diatoms [12, 20]) and recently *Cryptomonas* Φ (6) have been shown to contain divergent *rbcL* genes. In the latter organisms, both the small- and large-subunit genes are contained on the chloroplast genome, whereas other eukaryotes contain the *rbcS* gene on the nuclear genome (27). In prokaryotes, the large and small subunit are separated by a small noncoding region.

Our experiments with natural phytoplankton populations undoubtedly involved diverse microorganisms, and detection of absolute values of gene expression may have been underestimated. However, tropical oligotrophic waters are usually dominated by photosynthetic picoplankton, which are believed to contain *Synechococcus*-type organisms, which we would expect to easily detect with an *rbcL* probing system.

The control of RUBISCO expression is regulated by a myriad of mechanisms that vary among photosynthetic organisms, including the following: (i) activation of the enzyme by CO_2, (ii) enzyme activation by phosphorylated metabolites of the Calvin cycle, (iii) inhibition of RUBISCO by 2-carboxy arabinitol-1-phosphate in higher plants, (iv) activation of the enzyme by RUBISCO activase (eukaryotes only), (v) specific proteolytic digestion of RUBISCO (prokaryotes), and (vi) control of transcription of both *rbcS* and *rbcL* mRNA (27).

In general, the light induction of RUBISCO is caused by increases in both large- and small-subunit mRNAs (2, 23). In eukaryotes, this involves coordination of both chloroplast and nuclear genomes (24). In cyanobacteria, *rbcL* and *rbcS* are contranscribed, being separated by only a short spacer region (93 bp for *Synechococcus* PCC 6301 [23]).

Our preliminary findings suggest a light regulation of *rbcL* in natural phytoplankton consistent with that observed for most photosynthetic organisms (21–23, 26, 30). In our studies, the lowest levels of expression occurred at night (1100, 0300, and 0100 h), with greater levels during the day. The greatest variability in this trend was observed during the light/dark transition times of 0700 and 1900 h.

We did not quantitate the absolute amount of *rbcL* mRNA detected in *Synechococcus* cultures or environmental samples. Subsequent studies with *rbcL* mRNA standard curves indicated that the amount of hybridization detected could be between 0.2 and 0.5 ng of RNA per spot. Because each dot was 1/6th the total mRNA extracted, this represents between 1.8 and 3 ng of *rbcL* mRNA per sample.

The amount of hybridization detected in the *Synechococcus* culture (1-ml sample of 1.2×10^8 cells) was similar to that observed for mRNA extracted from 2 liters of seawater from the Dry Tortugas. We did not count picoplankton cells (or total phytoplankton abundance) in this sample but we did measure chlorophyll *a*. Chlorophyll *a* concentrations ranged from 0.19 to 0.36 μg/liter (0.38 to 0.72 per 2-liter sample). On the basis of an average cellular chlorophyll *a* content of 18 fg per cell for *Synechococcus* PCC 6301 (18a), we estimate that the amount of hybridization detected in our *Synechococcus* blots corresponded to a total chlorophyll *a* content of 2.2 μg per sample. Thus, an approximately equivalent mRNA signal was obtained for the natural phytoplankton samples and the *Synechococcus* culture, even though the former contained only 17 to 33% of the chlorophyll *a* contained by the latter. Because of potential sequence divergence of natural *rbcL* genes, we expected less detectable mRNA per unit of chlorophyll in picoplankton than for cultures of known genetic homology.

A problem with the diel study as performed is that it is not known if the same phytoplankton population was sampled at each time point. The sampling occurred from a moored vessel, and tidal currents may have brought in diverse populations over the sampling period. Vertical migration could have affected this study because samples were only taken at the surface. The general size of the phytoplankton population (as estimated by chlorophyll *a*) did not vary in a diel fashion, although there was a slight net increase in the chlorophyll *a* content over the study period. We have no means to assess variations in phytoplankton population diversity over the study period. Future studies will employ enclosed water columns or will track water masses marked with a drogue.

The procedures described herein should be applicable to detection of gene expression by genetically engineered organisms in the environment, organisms added for bioremediation purposes, or detection of conserved genes in indigenous microbial populations.

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REFERENCES

1. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:** 327–336.
2. **Berry, J. O., B. J. Nikolau, J. P. Carr, and D. P. Klessig.** 1985. Transcriptional and post-transcriptional regulation of ribulose 1,5-bisphosphate carboxylase gene expression in light- and dark-grown amaranth cotyledons. Mol. Cell. Biol. **5:**2238–2246.
3. **Capone, D. G., J. M. O'Neil, J. Zehr, and E. J. Carpenter.** 1990. Basis for diel variation in nitrogenase activity in the marine planktonic cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **56:**3532–3536.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium isothiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Cote, R.** 1984. ATCC media handbook, 1st ed. American Type Culture Collection, Rockville, Md.
6. **Douglas, S. E., D. G. Durnford, and C. W. Morden.** 1990. Nucleotide sequence of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Cryptomonas* Φ: evidence supporting the polyphyletic origin of plastids. J. Phycol. **26:**500–508.
7. **Ellis, R. J.** 1979. The most abundant protein in the world. Trends Biochem. Sci. **4:**241–244.

7a. **Fleming, J. T., and G. S. Sayler.** 1990. Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, Q-189, p. 320.

8. **Frischer, M. E., J. M. Thurmond, and J. H. Paul.** 1990. Natural plasmid transformation in a high-frequency-of-transformation marine *Vibrio* strain. Appl. Environ. Microbiol. **56**:3439–3444.
9. **Holm-Hansen, O., and B. Riemann.** 1978. Chlorophyll *a* determination: improvements in methodology. Oikos **30**:438–448.
10. **Huang, T.-C., and T.-J. Chow.** 1990. Characterization of the rhythmic nitrogen-fixing activity of *Synechococcus* sp. RF-1 at the transcriptional level. Curr. Microbiol. **20**:23–26.
11. **Huang, T.-C., T.-J. Chow, and J.-S. Hwang.** 1988. The cyclic synthesis of the nitrogenase of *Synechococcus* RF-1 and its control at the transcriptional level. FEMS Microbiol. Lett. **50**:127–130.
12. **Hwang, S.-R., and F. Tabita.** 1989. Cloning and expression of the chloroplast-encoded *rbcL* and *rbcS* genes from the marine diatom *Cylindrotheca* sp. strain N1. Plant Mol. Biol. **13**:69–79.
13. **Jeffrey, W. H., J. H. Paul, and G. J. Stewart.** 1990. Natural transformation of a marine *Vibrio* by plasmid DNA. Microb. Ecol. **19**:259–268.
14. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding for neomycin resistance. Mol. Gen. Genet. **177**:65–72.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Meyer, R., R. Laux, G. Boch, M. Hinds, R. Bayly, and J. A. Shapiro.** 1982. Broad-host-range IncP-4 plasmid R1162: effects of deletions and insertions on plasmid maintenance and host range. J. Bacteriol. **152**:140–150.
17. **Orellana, M. V., M. J. Perry, and B. A. Watson.** 1988. Probes for assessing single cell primary production: antibodies against ribulose-1,5-bisphosphate carboxylase (RuBPCase) and peridinin/chlorophyll *a* protein, p. 243–262. *In* C. M. Yentsch, F. C. Mague, and P. K. Horan (ed.), Immunochemical approaches to coastal, estuarine, and oceanographic questions. Springer-Verlag, New York.
18. **Paul, J. H.** 1982. The use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. **43**:939–949.
18a. **Pichard, S. L.** Unpublished data.
19. **Puissant, C., and L. M. Houdebine.** 1990. An improvement of the single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques **8**: 148–149.
20. **Reith, M. E., and R. A. Cattolico.** 1986. Inverted repeat of *Olithodiscus luteus* chloroplast DNA contains genes for both subunits of ribulose-1,5-bisphosphate carboxylase and the 32,000 dalton Q_B protein: phylogenetic implications. Proc. Natl. Acad. Sci. USA **83**:8599–8603.
21. **Sasaki, Y., T. Sakihama, T. Kamikubo, and K. Shinozaki.** 1983. Phytochrome-mediated regulation of two mRNAs, encoded by nuclei and chloroplasts of ribulose-1,5-bisphosphate carboxylase/oxygenase. Eur. J. Biochem. **133**:617–620.
22. **Sasaki, Y., Y. Tomoda, and T. Kamikubo.** 1984. Light regulates the gene expression of ribulose bisphosphate carboxylase at the levels of transcription and gene dosage in greening pea leaves. FEBS Lett. **173**:31–34.
23. **Shinozaki, K., Y. Sasaki, T. Sakihama, and T. Kamikubo.** 1982. Coordinate light-induction of two mRNAs, encoded in nuclei and chloroplasts, of ribulose 1,5-bisphosphate carboxylase/oxygenase. FEBS Lett. **144**:73–76.
24. **Shinozaki, K., and M. Sugiura.** 1983. The gene for the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase is located close to the gene for the large subunit in the cyanobacterium *Anacystis nidulans*. Nucleic Acids Res. **11**:6957–6964.
25. **Shinozaki, K., C. Yamada, N. Takahata, and M. Sugura.** 1983. Molecular cloning and sequence analysis of the cyanobacterial gene for the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase. Proc. Natl. Acad. Sci. USA **80**:4050–4054.
26. **Steinbiss, H. J., and K. Zetsche.** 1986. Light and metabolite regulation of the synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase and the corresponding mRNAs in the unicellular alga *Chlorogonium*. Planta **167**:575–581.
27. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. **52**:155–189.
28. **Tabita, F. R., and C. L. Small.** 1985. Expression and assembly of active cyanobacterial ribulose-1,5-bisphosphate carboxylase/oxygenase in *Escherichia coli* containing stoichiometric amounts of large and small subunits. Proc. Natl. Acad. Sci. USA **82**:6100–6103.
29. **Tsai, Y.-L., and B. H. Olson.** 1990. Effects of Hg^{2+}, Ch_3-Hg^+, and temperature on the expression of mercury resistance genes in environmental bacteria. Appl. Environ. Microbiol. **56**:3266–3272.
30. **Valle, E., H. Kobayashi, and T. Akazawa.** 1988. Transcriptional regulation of genes for plant-type ribulose-1,5-bisphosphate carboxylase/oxygenase in the photosynthetic bacterium, *Chromatium vinosum*. Eur. J. Biochem. **173**:483–489.
31. **Zehr, J. P., R. J. Limberger, K. Ohki, and Y. Fujita.** 1990. Antiserum to nitrogenase generated from an amplified DNA fragment from natural populations of *Trichodesmium* spp. Appl. Environ. Microbiol. **56**:3527–3531.