ELSEVIER

FEMS Microbiology Letters 201 (2001) 127–132

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

RNA recovery and detection of mRNA by RT-PCR from preserved prokaryotic samples

Dave S. Bachoon *, Feng Chen, Robert E. Hodson

Department of Marine Sciences, School of Marine Programs, University of Georgia, Athens, GA 30602, USA

Received 9 August 2000; received in revised form 6 October 2000; accepted 6 October 2000

First published online 18 June 2001

Abstract

The effectiveness of maintaining prokaryotic RNA in *Synechococcus* and *Pseudomonas* cells, fixed in 96% ethanol, 4% paraformaldehyde, or suspended in RNA*later*, and held in cold storage for 3 months was compared. Fluorometric determination of the RNA extracted from *Synechococcus* and *Pseudomonas* cells indicated that the cell storage treatments tested were equally effective at maintaining their total RNA content. There was not any detectable decrease in the quantity of RNA isolated from the preserved samples during storage. Intact mRNA transcripts of the RuBisCO (*rbcL*) and *nir* genes were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) from preserved bacterial cells throughout 3 months of storage. In contrast, RT-PCR failed to amplify the mRNA of the *rbcL* and nitrite reductase genes in unfixed and/or unpreserved bacterial samples, suggesting that bacterial mRNA can be well maintained during a prolonged storage when cells are preserved properly. In addition, RNA*later* is a useful reagent for the storage and maintenance of high quality RNA in unfrozen samples.

Keywords: Prokaryotic mRNA; RT-PCR; Preserved bacterial cell; RNA recovery

1. Introduction

The detection of prokaryotic ribonucleic acids (RNA) is increasingly used to analyze in a cultivation-independent way the structure of complex microbial communities [1,2]. Bacterial RNA analysis, usually involves small subunit ribosomal RNA (16S rRNA) and/or messenger RNA (mRNA). The unique signature sequence of bacterial 16S rRNA permits the development of oligonucleotide probes, which are used in phylogenetic identification of bacteria in complex microbial communities [1,3]; whereas, bacterial activity associated with specific gene expression can be monitored by detection of mRNA [4–7].

Specific bacterial RNA (i.e. rRNA and mRNA) can be detected from extracted bulk nucleic acids, or within individual cells. Typically, rRNA is detected inside cells by fluorescent in situ hybridizations (FISH) with rRNA-targeted oligonucleotide probes [8,9]. Because of the low copy number of specific mRNA molecules in prokaryotic cells [10], they are often amplified and detected by direct or indirect reverse transcriptase-polymerase chain reaction (in situ RT-PCR) [5,6,11,12]. These methods of prokaryotic RNA analyses are providing microbial ecologists with powerful tools to investigate the links between the abundance and spatial distribution of bacteria and their in situ physiological activities.

Accurate estimation of bacterial abundance, diversity and activity by in situ RNA methodologies rely on the presence of bacterial cells with intact RNA molecules. Therefore, it is important to know how to fix and store microbial samples for later analysis. It has been reported that prokaryotic mRNA has a much shorter half life compared with prokaryotic rRNA [4,13,14] and in dead bacterial cells degraded to undetectable levels after 2–16 h at room temperature. In contrast, 16S rRNA transcripts remained intact, and were detected by RT-PCR in dead cells kept at room temperature for 16 h [15], suggesting that 16S rRNA is more stable compared to mRNA inside bacterial cells. The persistence of RNA molecules in stored bacterial samples depends on the method of cell fixation and subsequent holding conditions [15]. Reported cell fixation time varies from 30 min to several days [6,16]. Although previous studies suggest less than 48 h should be used for bacterial fixation in 4% paraformaldehyde (PFD) due to the potential of crosslinking between RNA and other cellular components, little is known about the

* Corresponding author. Tel.: +1 (706) 542-5125; Fax: +1 (706) 542-5888; E-mail: dbachoon@arches.uga.edu

0378-1097/01/$20.00
PII: S0378-1097(01)00251-8

recovery yield or the stability of prokaryotic RNA from fixed and stored cells.

In this study, we evaluated several methods of prokaryotic cell fixation and storage for maintaining RNA molecules suitable for molecular analyses, after which the isolated RNA from these cells was quantified by fluorescence staining, while RNA transcripts (16S rRNA, mRNA (Rubisco 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) or nitrite reductase (*nirS*))), in respective samples, were detected by RT-PCR.

2. Materials and methods

2.1. Bacterial samples and cell preservation

One strain of photoautotrophic cyanobacteria *Synechococcus* sp. and a heterotrophic denitrifying *Pseudomonas stutzeri* (ATTC 14405) were used in this study. *Synechococcus* strain 7803 was grown under continuous light in SN media [17], at 26°C and 16–18 μmol of quanta m^{-2} s^{-1}. *P. stutzeri* (ATTC 14405) was grown in marine broth (Sigma) under oxygen limited conditions. All cultures were grown to exponential phase and had an approximate cell density of 2×10^8 cells ml^{-1} as determined by acridine orange direct counts (AODC) [18]. Cells were harvested from 200 ml of bacterial culture by centrifugation at $3000\times g$ for 10 min at 4°C and washed twice with ice chilled diethyl pyrocarbonate (DEPC) (0.1%) treated phosphate buffered saline (PBS; 120 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffered (pH 7.6). These cells were resuspended in 6 ml of PBS (DEPC treated) and kept on ice for 5–10 min until fixation and storage. 100 μl of this cell suspension was used for an initial RNA extraction.

Subsamples of cell concentrates (700 μl) from each culture were treated and stored as follows: (1) frozen at −70°C; (2) frozen at −20°C in 50% ethanol (ethanol/PBS, 1:1); (3) suspended in 5 volumes of *RNAlater* (Ambion, Austin, TX, USA) at 4°C; (4) fixed in 4% fresh paraformaldehyde in PBS (ice chilled) for 15 min, 30 min, 1 h, and 2 h, respectively. Cells resuspended in paraformaldehyde were washed twice in PBS using centrifugation and stored in 50% ethanol (ethanol/PBS, 1:1) at −20°C.

2.2. RNA isolation

The samples stored at −70°C were thawed on ice prior to the removal of cells for RNA extraction and then immediately refrozen. Cells preserved in *RNAlater* or 50% ethanol did not solidify and were sampled at their specified storage temperatures. All buffers and solutions used for RNA extraction were treated with DEPC to inhibit the activity of RNase [19]. RNA was extracted biweekly from 100 μl portions of the preserved cells using the RNA extraction kit and following the procedure in the RNeasy Mini Handbook (Qiagen, Santa Clarita, CA, USA) with minor modifications. Cells were washed twice in PBS buffer and resuspended in 100 μl of lysis buffer (100 mM Tris, 50 mM EDTA (pH 8.0)). Cell lysis was achieved by treatment with proteinase K at a final concentration of 0.20 μg ml^{-1} for 15 min and lysozyme at a final concentration of 1.0 mg ml^{-1} for 30 min at room temperature after which 350 μl of RLT buffer (RNeasy kit) was added to the mixture and stored on ice. The cell lysates were mixed with 300 μl of acid phenol and incubated at 65°C for 10 min, followed by the addition of 300 μl of chloroform. Cell lysates were centrifuged at $3000\times g$ for 5 min and the aqueous phase was removed and added to 250 μl of ethanol at room temperature. The remainder of the RNA extraction procedure followed the protocol described in the RNeasy Mini Handbook (Qiagen). Isolated RNA was immediately stored at −70°C until used. An aliquot (5 μl) was examined by gel electrophoresis in 1.2% formaldehyde agarose gels stained with ethidium bromide and viewed with a UV transilluminator (RNeasy Mini Handbook, Qiagen).

2.3. Nucleic acid quantification

In order to quantify RNA or DNA concentration respectively, nucleic acid was subjected to DNase or RNase treatment prior to fluorescent measurements. 5 μl aliquots of nucleic acids were pretreated for 2 h at room temperature with 5 units of RNase-free DNase in 5 μl of digestion buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 2 mM $CaCl_2$) or 5 units of DNase-free RNase in 5 μl of digestion buffer to eliminate their respective contaminating DNA or RNA. Residual DNase from the RNA samples was heat inactivated at 80°C for 5 min [15] prior to RT-PCR amplification. The concentration of DNA, RNA and total nucleic acids was determined by staining 2 μl nucleic acid samples with the fluorescent dye RiboGreen following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA) and using a Turner Fluorometer (TD 700).

2.4. One tube RT-PCR

At the end of 3 months, DNase treated RNA from *Synechococcus* was used for RT-PCR to amplify a *rbcL* mRNA fragment and DNase treated RNA from *P. stutzeri* was used to amplify a *nirS* gene fragment. In addition, PCR was performed on the DNase treated RNA isolated from each of the preserved sample treatments to check for any contaminating DNA by using the Titan One Tube RT-PCR system (Boehringer, Mannheim, Germany) and 16S rDNA primers 27F (5′-AGA GTT TGA TCM TGG CTC AG) and 338R (5′-GCT GCC TCC CGT AGG AGT) [20]. For PCR, the reverse transcriptase (AMV) activity was inactivated by hot starting the Titan RT-PCR reaction mixture at 95°C for 5 min. DNase treated

RNA (approximately 100 ng) was amplified using a PTC-200 Thermal cycler (MJ Research) with 10 μM primer pairs, 1 U of Titan enzyme mix, 1× RT-PCR buffer (1.5 mM $MgCl_2$), 5 U RNase inhibitor, 5 mM dithiothreitol (DTT), and 100 μM each deoxynucleotide triphosphate in a reaction volume of 40 μl. The reaction mixtures were subjected to amplification for 40 cycles of 94°C for 30 s, 55°C for 1.5 min, 68°C for 45 s and a final cycle of 68°C for 8 min.

RT-PCR amplification of the DNase treated RNA samples was performed using the conditions described for PCR amplification with the following exceptions: (1) A 579 bp fragment of the *rbcL* gene was amplified from *Synechococcus* RNA with the primers WH*BCL*1 (5′-CTGCTTCAAGTGCACCGG) and WH*BCL*2 (3′-TTCGACAGGCTCGTCCT), which are specific for the *rbcL* gene of many phycoerythrin containing *Synechococcus*. A *nirS* gene region of 721 bp from *Pseudomonas* RNA was amplified using the primers *nirS* A (5′-CGCCAGAGTTCTCCCTGCAG) and *nirS* B (3′-CAGCTGTGGTTCTGGCCGTT) [21]. (2) Reverse transcription was performed at 55°C for 30 min to synthesize cDNA. (3) The hot start at 95°C was eliminated. Samples of each reaction (5 μl) were analyzed by gel electrophoresis (2% agarose as before). Representative samples of PCR product amplicons from the *rbcL* gene and *nirS* genes were purified with the Wizard DNA clean-up system (Promega, Madison, WI, USA) and sequenced with an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The final concentration of amplicons in each sample was determined by fluorometric measurements with PicoGreen (Molecular Probes) as previously described.

3. Results and discussion

It is common practice to fix prokaryotic cells in 96% ethanol or 4% buffered paraformaldehyde and store frozen until performing RNA analysis [22,23]. However, limited information is available on the persistence of bacterial RNA inside cells under different fixation and storage conditions. Presumably methods of cell fixation and storage should inactivate or inhibit the activity of RNases and maintain intact RNA (rRNA and mRNA) for in situ molecular analyses.

Previous work has demonstrated that an effective method of cell lysis is critical for the efficient extraction of nucleic acid from bacterial samples [24]. The method of cell fixation influenced the proportion of cell lysis as determined by AODC; cells fixed in 96% ethanol or preserved in *RNAlater* readily lysed after enzymatic pretreatments with 1.0 mg ml^{-1} lysozyme (final concentration) at room temperature for 30 min (data not shown), whereas cells which were fixed in 4% paraformaldehyde for 2 h were the most resistant to lysis. Treating the cells with 0.20 μg ml^{-1} proteinase K (final concentration) for 15 min and lysozyme for 30 min to weaken the cell walls increased cell lysis to more than 98% (data not shown). These results are consistent with previous reports by Wagner et al. [22], which demonstrated that bacterial cells fixed in 4% paraformaldehyde were more resistant to cell wall permeabilization than cells fixed in 96% ethanol. After cell lysis, the RNA from all lysates was recovered using the RNeasy Mini Kit. Nuclease (RNase and DNase) digestion of the recovered RNA samples, followed by analysis using agarose gel electrophoresis showed fluorescent bands consistent with the presence of RNA molecules.

Total RNA concentration from preserved cells, over 3 months of storage, was determined using the RiboGreen RNA quantification reagent which accurately measures RNA concentration in the presence of contaminants commonly found to contaminate nucleic acids including nucleotides, salts, ethanol, proteins and agarose (Molecular Probes). Twice as much RNA was recovered from the *Pseudomonas* cells compared with the *Synechococcus* cells (Table 1). Regardless of the cell fixation and storage conditions, the quantities of total RNA recovered from within each bacterial sample were similar.

Table 1
Fluorometric quantification of total RNA (ng μl^{-1}) isolated from 100 μl *P. stutzeri* and *Synechococcus* strain WH7803 cell concentrate that were fixed and stored for up to 3 months

Treatments	Mean RNA concentration (ng μl^{-1})			
	P. stutzeri		WH7803	
	6 weeks	12 weeks	6 weeks	12 weeks
PBS (−70°C)	423 (13)	400 (16)	176 (15)	168 (10)
RNAlater (4°C)	422 (9.3)	414 (12)	171 (9)	180 (12)
96% ethanol (−20°C)	408 (15)	397 (11)	173 (17)	168 (14)
15 min PFD (−20°C)	421 (11)	396 (15)	177 (19)	174 (12)
30 min PFD (−20°C)	401 (20)	395 (13)	180 (13)	169 (17)
1 h PFD (−20°C)	398 (12)	393 (10)	168 (13)	166 (21)
2 h PFD (−20°C)	401 (14)	390 (8)	165 (12)	174 (15)

RNA was extracted from cell concentrates, after 6 and 12 weeks. Prior to cell preservation the RNA concentration of the *P. stutzeri* and *Synechococcus* strain WH7803 cells were 420 ± 16 and 184 ± 22 ng μl^{-1}, respectively. Values in brackets are standard deviations of the mean ($n = 3$).
PBS, phosphate buffered saline, PFD, paraformaldehyde.

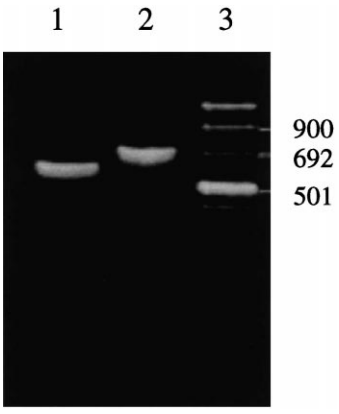

Fig. 1. RT-PCR fragments amplified from extracted RNA of (lane 1) *Synechococcus* sp. WH8101 *rbcL* gene using the primer pair WHR*BCL*1–WHR*BCL*2 and (lane 2) *P. stutzeri nirS* gene using the primer pair *nirS* A–*nirS* B. Lane 3 are fragments of lambda molecular mass marker VIII. DNA fragments were separated by electrophoresis in 2% agarose and stained with ethidium bromide.

The results in Table 1 suggest that the bacterial RNA from fixed cells (ethanol or paraformaldehyde), stored in *RNAlater* at 4°C or PBS at −70°C was detectable by fluorometric analysis. In addition, all of the storage procedures examined in this study adequately maintained bacterial cellular RNA content as determined with RiboGreen. Although fluorometric analysis of RNA provided useful quantitative information, it could not discriminate rRNA, tRNA, mRNA molecules and intact or partially degraded RNA molecules. Depending on their physiological state, rRNA and tRNA contribute >95% of the total bacterial RNA, and mRNA contributes 0–5% of the cellular RNA [10,25]. Moreover, mRNA decays much faster than rRNA or tRNA [15,25]. Sheridan et al. [15] reported that in dead cells held at room temperature, mRNA became undetectable after 2 h by RT-PCR; in contrast, 16S rRNA was detected in these cells after 16 h. It is reasonable to assume that if RNA degradation occurred in the stored bacterial cells, then mRNA would have degraded faster than rRNA or tRNA. The resulting change in total isolated RNA concentration as determined by fluorometric RNA measurement would conceivably have been undetected because bacterial mRNA constitutes a small portion of the total cellular RNA content and partially degraded RNA molecules remain detectable by fluorometry. Consequently, more meaningful information on the stability of *Pseudomonas* and *Synechococcus* RNA in stored samples was obtained by using RT-PCR analysis tools to monitor intact mRNA molecules.

RT-PCR has been used to detect prokaryotic mRNA in vitro or in situ [5,26]. Usually, as a precaution against false positive results, before performing RT-PCR, RNA samples are pretreated with RNase-free DNase to eliminate any DNA contaminants [4,5]. The absence of PCR amplicons confirmed that the DNase treated RNA was free of DNA contamination and that the RNA samples were suitable for use in RT-PCR reactions. RNA isolated from preserved *Synechococcus* WH7803 and *P. stutzeri* cells were amplified by RT-PCR with their respective primers and produced amplicons of the expected size (Fig. 1). The amplicon sequences were identical to the partial sequence of the *rbcL* or nitrite reductase gene.

The concentrations of the RT-PCR amplicons from RNA extracts of preserved *P. stutzeri* and *Synechococcus* sp. WH7803 are shown in Table 2. The concentrations of RT-PCR products amplified from isolated mRNA of preserved cells were not significantly different in comparison to that of their respective initial unfixed cells, suggesting that cellular mRNAs were well preserved using the methods described above. However, RT-PCR failed to yield detectable products against RNA extracted from unfixed *Pseudomonas* and *Synechococcus* cells held at room temperature for 24 h (Table 2), suggesting that the mRNA degraded quickly in unpreserved cells. In contrast, mRNA was detected in RNA extracted from cells which were stored at room temperature in *RNAlater* for 24 h (Table 2), indicating that the integrity of the mRNA molecules in cells stored in *RNAlater* was well maintained. Because the concentration of amplicons in any PCR is dependent on the initial template concentration, we assumed that the concentrations of mRNA recovered from preserved bacterial cells with different treatments were probably similar. However more reliable estimates of mRNA concentration from the preserved cells require the use of quantitative RT-PCR. Furthermore, the *rbcL* and *nirS* transcripts amplified by RT-PCR were intact, at least for the regions

Table 2
Fluorometric quantification of *nirS* amplicons from *P. stutzeri* and *rbcL* amplicons from *Synechococcus* strain WH7803 amplified by RT-PCR from 100 ng of isolated RNA from cells that were fixed and stored for 3 months

Treatments	Mean amplicon concentration (ng μl^{-1})	
	P. stutzeri	WH7803
Initial[a]	8.8 (2.1)	3.6 (1.1)
PBS at RT (24 h)[b]	n.d.	n.d.
PBS (−70°C)	6.0 (2.2)	2.7 (1.5)
RNA*later* at RT (24 h)[c]	5.8 (1.2)	2.5 (0.5)
RNA*later* (4°C)	6.3 (1.8)	2.7 (0.9)
96% ethanol (−20°C)	7.1 (1.3)	2.8 (0.6)
15 min PFD[d] (−20°C)	7.3 (2.4)	3.1 (1.6)
30 min PFD (−20°C)	6.6 (1.0)	3.2 (1.1)
1 h PFD (−20°C)	6.2 (1.7)	3.7 (0.5)
2 h PFD (−20°C)	5.5 (2.3)	2.8 (0.9)

Values in brackets are standard deviations of the mean ($n = 3$).
n.d., not detected.
[a]Initial samples were not stored, RNA was extracted from 100 µl of cell concentrate at time zero and 100 ng of RNA was used in RT-PCR.
[b]RNA extracted from cell concentrate in PBS kept at room temperature for 24 h.
[c]RNA extracted from cell concentrate in RNA*later* kept at room temperature for 24 h.
[d]PFD, paraformaldehyde.

along their respective primer (WH*BCL* or *nir*) binding sites. These results suggest that bacterial mRNA from *P. stutzeri* and *Synechococcus* cells is stable during prolonged storage when samples are fixed, preserved and stored by a variety of methods. However, these methods may not be universally applicable for preserving prokaryotic mRNA in bacterial cells from environmental samples that have grossly different cell wall structure and lower mRNA copy numbers from those of laboratory cultures.

The method of prokaryotic cell fixation and storage for molecular analysis may vary depending on the type of experiment or field investigation. Our study indicated that bacterial mRNA was stable in *RNAlater* at room temperature for 24 h, and in RNA*later* at 4°C for several months. These results were consistent with previous reports, which demonstrated that *Escherichia coli* cells stored in *RNAlater* for 1 month at 4°C yielded undegraded RNA and intact RNA was isolated from tissue samples stored for 1 week at 25°C (Ambion; *RNAlater*™). Therefore, *RNAlater* might be ideal for stabilizing bacterial rRNA and mRNA in unfrozen samples, for prolonged shipment or field studies. In this study, we observed that the length of time used to fix the bacterial cells in paraformaldehyde did not affect the quantity or quality of the RNA isolated. However, increased duration of cell fixation in paraformaldehyde increased the resistance of the cells to enzymatic permeabilization. Molecular techniques such as FISH or in situ RT-PCR require cell wall permeabilization, just enough, to allow oligonucleotide probes to enter the cell, but not as much to cause cell lysis. Consequently, we suggest that for FISH or in situ PCR, bacterial cells should be fixed in 4% paraformaldehyde for 1–2 h and stored at −20°C to maintain cell wall integrity and cellular RNA content. For RNA analysis by techniques such as Northern blotting, bacterial cells could be stored in PBS at −70°C, 50% ethanol at −20°C or in *RNAlater* at 4°C.

In this study, we demonstrated that prokaryotic mRNA from laboratory cultures could be preserved in various fixation and storage treatments for in vitro and in situ molecular analyses. RT-PCR of the isolated bacterial RNA was an effective method for the detection of *rbcL* transcripts from *Synechococcus* cells, and *nirS* transcripts from *Pseudomonas* cells. For field studies, *RNAlater* could be useful for the storage and maintenance of high quality RNA in unfrozen samples.

Acknowledgements

This work was funded by the following US agencies: Department of Energy (DE-FG02-97ER62451), National Science Foundation (OCE-9730602), NOAA/Sea Grants (NA66RG0282), and the NSF LMER program (DEB-9412089). We thank Dr. Jingrang Lu and Jim Sullivan for providing us with the bacterial cultures.

References

[1] DeLong, E.F., Wickham, G.S. and Pace, N.R. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.
[2] Giovannoni, S.J., Delong, E.F., Olsen, G.J. and Pace, N.R. (1988) Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. J. Bacteriol. 170, 720–726.
[3] Amann, R.I., Binder, B.J., Olson, R.J., Chisolm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial population. Appl. Environ. Microbiol. 56, 1919–1925.
[4] Oelmuller, U., Kruger, N., Steinbuchel, A. and Friedrich, C.G. (1990) Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods 11, 73–84.
[5] Hodson, R.E., Dustman, W.A., Garg, R.P. and Moran, M.A. (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl. Environ. Microbiol. 6, 4074–4082.
[6] Tolker-Nielsen, T., Holmstrom, K. and Molin, S. (1997) Visualization of specific gene expression in individual *Salmonella typhimurium* cells by *in situ* PCR. Appl. Environ. Microbiol. 63, 4196–4203.
[7] Chen, F., Dustman, W.A. and Hodson, R.E. (1999) Detection of toluene dioxygenase gene and gene expression in *Pseudomonas putida* F1 in a toluene exposed seawater using *in situ* PCR and hybridization. Hydrobiologia 401, 131–138.
[8] Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schliefer, K.-H. (1994) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792–800.
[9] Wagner, M., Rath, G., Amann, R., Koops, H.-P. and Schliefer, K.-H. (1995) *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 17, 251–264.
[10] Kramer, J.G. and Wyman, M. (1998) In: Molecular Approaches to the Study of the Ocean (Cooksey, K.E., Ed.), pp. 131–145. Chapman-&Hall, London.
[11] Chen, F., Gonzalez, J.M., Dustman, W.A., Moran, M.A. and Hodson, R.E. (1997) *In situ* reverse transcription: an approach to characterize genetic diversity and activities in prokaryotes. Appl. Environ. Microbiol. 63, 4907–4913.
[12] Chen, F., Binder, B. and Hodson, R.E. (2000) Flow cytometric detection of specific gene expression in prokaryotic cells using in situ RT-PCR. FEMS Microbiol. Lett. 184, 291–295.
[13] Holmstrom, K., Tolker-Nielsen, T. and Molin, S. (1999) Physiological states of individual *Salmonella typhimurium* cells monitored by reverse transcription-PCR. J. Bacteriol. 181, 1733–1738.
[14] Belasco, J. and Higgns, C. (1988) Mechanisms of mRNA decay in bacteria: a perspective. Gene 72, 15–23.
[15] Sheridan, G.E., Masters, C.C.I., Shallcross, J.A. and Mackey, B.M. (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. 64, 1313–1318.
[16] Hahn, D., Amann, R.I. and Zeyer, J. (1993) Detection of mRNA in Streptomyces cell by whole-cell hybridization with digoxigenin-labeled probes. Appl. Environ. Microbiol. 59, 2753–2757.
[17] Waterbury, J.B., Watson, S.W., Valois, F.W. and Franks, D.G. (1986) Biological and ecological characterization of the marine unicellular cyanobacteria *Synechococcus*. Can. Bull. Fish. Aquat. Sci. 214, 71–120.
[18] Hobbie, J.E., Daley, J. and Jasper, S. (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33, 1225–1228.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[20] Suzuki, M.T. and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625–630.
[21] Ward, B.B., Cockcroft, A.R. and Kilpatrick, K.A. (1993) Antibody and DNA probes for detection of nitrite reductase in seawater. J. Gen. Microbiol. 139, 2285–2293.
[22] Wagner, M., Schmid, M., Juretschko, S., Trebesius, K.-H., Bubert, A., Goebel, W. and Schleifer, K.-H. (1998) In situ detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*. FEMS Microbiol. Lett. 160, 159–168.
[23] Hahn, D., Amann, R.I., Ludwig, W., Akkermans, A.D.L. and Schleifer, K.H. (1992) Detection of microorganisms in soil after *in situ* hybridization with rRNA targeted fluorescently labeled oligonucleotides. J. Gen. Microbiol. 138, 879–887.
[24] More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C. and Madsen, E.L. (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl. Environ. Microbiol. 60, 1572–1580.
[25] Arraiano, C.M. (1993) Post-transcriptional control of gene expression: bacterial mRNA degradation. World J. Microbiol. Biotech. 9, 421–432.
[26] Chen, F., Gonzalez, J.M., Dustman, W.A., Moran, M.A. and Hodson, R.E. (1998) *In situ* PCR methodologies for visualization of microscale genetic and taxonomic diversities of prokaryotic communities. In: Molecular Microbial Ecology Manual (Akkermans, A.D.L., van Elsas, J.D. and DeBruijn, F.J., Eds.), Ch. 3.39, pp. 1–17. Kluwer Academic Publishers, Dordrecht.