Application of *In Situ* Reverse Transcription to Estuarine Bacterial Community Analysis

F. Chen[1], W.A. Dustman[2], R.E. Hodson[1, 2, 3]

[1]Department of Marine Sciences, University of Georgia, Athens, Georgia 30602-2206
[2]Department of Microbiology, University of Georgia, Athens, Georgia 30602-2206
[3]Institute of Ecology, University of Georgia, Athens, Georgia 30602-2206

ABSTRACT

Bacterial communities along a salinity gradient (0 to 30 ‰) of the Satilla River in southern Georgia were analyzed by *in situ* reverse transcription (ISRT) using primers specific for the beta, gamma, delta, marine alpha proteobacteria, subdivisions of eubacteria, as well as one for eubacterial domain. Bacteria yielding a positive ISRT reaction with the eubacterial domain primer accounted for 76, 43, and 85% of total DAPI-positive cells at stations with salinities of 34.8, 24.6, and 0‰, respectively. Marine alpha-positive bacteria were most numerous (32 % of eubacteria) at the highest salinity and were least abundant (<1%) in freshwater stations. The gamma *Proteobacteria* were more uniformly distributed and accounted for 23 to 42% eubacteria from the freshwater end to the coastal site of the river. The high percentage of gamma *Proteobacteria* across a broad range of salinities may be associated with high abundance of detrital material in the coastal river system. The beta *Proteobacteria* were more abundant (41% of eubacteria) near the freshwater end and less abundant (13% eubacteria) in marine-type samples. Delta-proteobacteria accounted for less than 8% of eubacteria at all salinities examined. ISRT was also used to detect the proportion (12-30%) of the marine bacterial community that actively expressed genes for degradation of aromatic hydrocarbons (i.e. benzene, ethanol, toluene and xylene). In unamended estuarine water, no bacteria expressing genes for degradation of the hydrocarbons were detected.

Introduction

For many years, microbial ecologists have been seeking tools to closely examine the structure and function of microbial communities in natural marine environments where <1 % of the microorganisms are cultivable. Using recently developed molecular techniques based on extraction of microbial nucleic acids followed by PCR amplification, DNA hybridization, cloning and sequencing, we are now able to detect specific microorganisms in natural marine environments, and to study their genetic diversity and phylogenetic kinship [10, 11, 13, 27].

In situ identification of individual marine microbes based on their genetic characteristics is another important application that has significantly advanced our understanding of microbial community structure and function in various microenvironments [8]. Whole-cell immunofluorescence assay [4, 26], fluorescent *in situ* hybridization (FISH) based on 16S rRNA sequences [2, 9, 20], and chromosomal painting [21] techniques all have been used to characterize specific bacterial strains or populations in complex microbial communities.

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (ed)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

Additionally, prokaryotic *in situ* PCR and *in situ* RT-PCR protocols have been developed to taxonomically identify and visualize the presence of genes and their expression inside individual bacterial cells [5, 6, 18, 22, 25]. PCR *in situ* detection methods amplify specific targets intracellularly and thus can detect low or even single-copy number of sequences in DNA, mRNA or rRNA. Therefore, *in situ* amplification techniques can be used to explore genetic capabilities and activity, as well phylogenetic affinities of individual microbes at microscale levels.

In situ reverse transcription (ISRT) is a simplified *in situ* molecular tool that has shown great potential for analyzing the expression of functional genes in individual cells. ISRT using radioactively labeled probes was first used to detect mRNA in fixed animal tissue sections (23), and was developed to detect prokaryotic cells based on their rRNA and mRNA sequences by Chen et al. [7]. Detection of gene expression using FISH has been reported using a system in which very long probe (e. g., 200 bases) are prepared using in vitro transcription [16, 19]. FISH with long multi-labeled probes [24] has been demonstrated to increase signal intensity relative to FISH using mono-labeled probes. However, in studies of natural systems where many probes need to be applied, FISH with very long probes or multiple probes can be very time-consuming, and thus impractical. ISRT takes advantage of multiple label incorporation into multiple copies of RNA molecules inside bacterial cells providing a high sensitivity for phylogenetic identification of bacterial cells in natural populations as well as detection of cells expressing a specific functional gene within complex bacterial communities. With ISRT only a typical antisense PCR primer (unlabeled) is needed. Long labeled transcripts are formed intracellularly by incorporation of labeled monomers.

In this study, we applied ISRT to investigate microscale distribution of several major groups of bacteria from a river to a coastal zone in Georgia. In addition, ISRT was also used to examine how natural microbial communities respond to exposure of monoaromatic contaminants.

Methods and Materials

Sample preparation

Bacterial samples were collected from three stations (Station 1, 3 and 6) along the Satilla River in July 12, 1998 (Fig. 1). The salinity at Stns.1, 3, and 6 were 34.8, 24.6, and 0‰, respectively. Samples were fixed in 4% paraformaldehyde and used for ISRT analysis within 48 hr. Bacteria in natural samples were concentrated by centrifugation. A 1.5 ml water sample was centrifuged at 13,000 rpm for 10 min and the pellet resuspended in 15 µl of PBS buffer. An additional 15 µl of ethanol (98%) was added to the resuspended sample. Samples stored at this stage were found to be stable for up to 2 months if kept at 4 °C. Bacterial cell pretreatment, including cell wall permeabilization and dehydration, followed the methods described by Hodson et al. [18]. Water, buffer and washing solutions were pre-treated with diethyl pyrocarbonate (DEPC) to remove RNase that may degrade cellular RNA, especially mRNA.

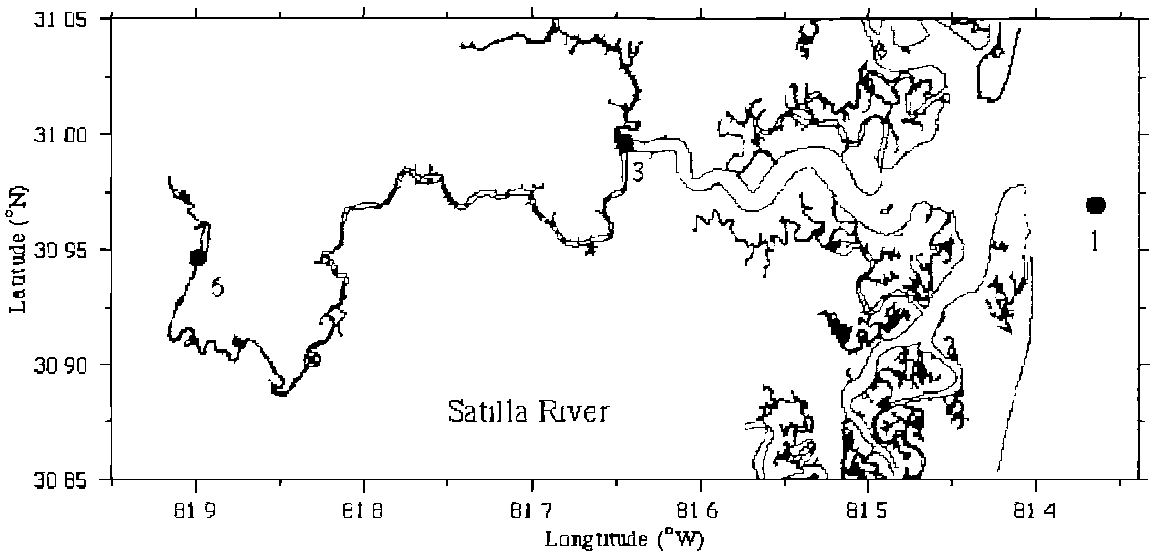

Fig. 1. Map of the Satilla River showing the three sampling sites (Station 1, 3, and 6).

Toluene/xylene exposure

A 50 ml sample of seawater, collected from Meridian Dock near Sapelo Island, Georgia, was added to each of three flasks. The first flask was exposed to 0.2% toluene, the second flask to 0.2% xylene, and the third flask served as a control. The samples were incubated at room temperature for one week and then concentrated as described above.

Primers for 16S rRNA and functional genes

To achieve a sufficient incorporation of labeled nucleotides into cDNA, a primer located at least 200 bases downstream to the 5' end of the RNA sequence is required for ISRT. Bacterial community composition was analyzed using ISRT with primers specific for the beta, gamma, delta, or marine alpha proteobacterial groups (Table 1). Microbial response to exposure to aromatic compounds (toluene or xylene) was examined by ISRT using a primer specific for BTEX (benzene, toluene, ethanol and xylene) dioxygenases, 5'ACCCAGTTCTC(G/C)CC(G/A)TC(G/A)TCCTG3'. The BTEX primer was designed based on the sequence alignment of 12 different BTEX dioxygenase genes.

Table 1. Sequences and locations of bacterial group-specific primers used in this study.

Primer Specificity	Primer Sequence	Location	Reference
Marine Alpha	5' GCCGGGGTTTCTTTACCA	16S, 488-507	[15]
Beta	5' GCCTTCCCACTTCGTTT	23S, 1027-1043	[3]
Gamma	5' GCCTTCCCACATCGTTT	23S, 1027-1043	[3]
Delta	5' CGGCGTCGCTGCGTCAGG	16S, 385-402	[3]
Eubacteria	5' ACCGCTTGTGCGGGCCC	16S, 927-942	[3]

ISRT

ISRT was conducted according to the protocols described by Chen et al. [7]. Briefly, after cell fixation and permeabilization, the primer was hybridized to the RNA target site for two hr using the protocol described by Zarda et al. [28]. Unbound primers on slides were washed off with 0.5X SSC solution (10 min at 45 ^{o}C). Samples on slides were dehydrated sequentially in 50, 80 and 98% ethanol in PBS. The ISRT cocktail consisted of 1X enzyme buffer, 200 µM each of dATP, dCTP, dGTP, 190 µM dTTP, 10 µM CY3-dUTP, RNase inhibitor (0.8 U µl^{-1}, Boehringer Mannheim), 0.02% bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), and thermostable r*Tth* reverse transcriptase (Perkin Elmer) (1 U µl^{-1}). The cell spots were then covered with 40 µl of reverse transcription mixture and slides were incubated at 60 ^{o}C for 1 hr in a humid chamber. Samples on slides were then counterstained with DAPI at the concentration of 0.5 µg ml^{-1} for 5 min. Slides were washed once with distilled water and air dried. Samples on slides were mounted in Olympus immersion oil.

Digital image analysis

Slides were examined under an epifluorescence microscope (Olympus BX-40). Digital images were acquired with a cooled CCD camera (Photometrics, Tucson, Ariz.). Image analysis was processed using the Oncor Image software package version 2.02 (Oncor, Inc., Gaithersburg, Maryland) on a Power Macintosh 9500. Target cells labeled with CY3 were viewed under orange light, while total bacterial cells counterstained with DAPI (in the same viewfield) were viewed under UV light. Data presented in this study are mainly expressed as the relative proportion of target cells vs. total DAPI stained cells, or target cells vs. total cells giving positive ISRT reaction using a eubacterial primer. At least 300 cells were counted for each group of bacteria, and two to three replicates were counted for each sample. Autofluorescence background caused by cyanobacteria and other chlorophyll containing particles was determined by inspecting sample spots not hybridized with group-specific probes.

Results and Discussion

During our initial trials, ISRT was tested against samples collected on 0.2 µm pore-size black membrane filters. We found it was difficult to define real positive signals due to a high background of CY3 fluorescence. Even with highly stringent washes, we were still not able to remove the background fluorescence from filters. When ISRT was tested using concentrated samples spotted onto glass slides, the background fluorescence was significantly reduced. Using this method even small bacterial cells with low positive signals can still be seen on a dark background. Therefore, in this preliminary study, all ISRT analyses were conducted using samples concentrated by centrifugation and applied to slides. The recovery efficiency of cells remaining on the slide throughout the ISRT procedures was generally greater than 90%. Ideally, conducting *in situ* molecular detection using samples on filters should be the best way to visualize microscale distribution of bacteria associated with their microhabitats. Currently, we are conducting tests using ISRT against bacterial samples collected on different types of filters.

Bacterial communities at the three stations looked quite different in terms of morphology and size of bacteria. Most of the bacteria at Stn. 1 were small cocci-shaped cells; most cells at Stn. 3 were attached to particles or debris; and the population at Stn. 6 was a mixture of cocci and rod-shaped cells. There was a notable shift toward larger and more elongate cells from high salinity to low salinity waters. In all samples, autofluorescent cells or particles accounted for <2% of total DAPI counts. Cells detected by ISRT with the eubacterial primer (EUB927) accounted for 76, 43 and 85% of total DAPI-stained cells at Stns. 1, 3, and 6, respectively (Table 2). Previous reports based on FISH found that eubacteria account for 35 to 67% of DAPI counts in natural bacterioplankton communities [17]. Samples from Stn. 3 contained high levels of detritus and higher fluorescent backgrounds, probably resulting in underestimates of cell numbers by ISRT.

The relative proportion of each of the major taxonomic subgroups varied between Stns. 1, 3, and 6. Marine alpha *Proteobacteria* accounted for 32 and 18 % of total eubacteria at Stns. 1 and 3, respectively (Table 2), but <1% of eubacteria in the freshwater of Stn. 6. This observation is in concordance with the distribution of marine alpha bacteria in Georgia coastal rivers as determined by *in vitro* hybridization [15]. The marine alpha proteobacterial group was first defined by Gonzalez and Moran [15] based on the close phylogenetic kinship among a group of alpha *Proteobacteria* isolated from marine environments. They found that the percentage of marine alpha *Proteobacteria* declines with decreasing salinity along an estuarine transect and the group was not detectable in freshwater samples.

Table 2. Proportion of each bacterial group vs. eubacteria in the three sampling stations.

Bacterial groups	**Stn. 1 (34.8‰)**	**Stn. 3 (24.6‰)**	**Stn. 6 (0‰)**
Eubacteria*	76%	43%	85%
Marine Alpha	32%	18%	<1%
Gamma	33%	42%	23%
Beta	13%	19%	41%
Delta	5%	8%	7%

The beta *Proteobacteria* accounted for 13%, 19% and 41% of eubacteria at Stns. 1, 3 and 6, respectively, showing an apparent affinity for freshwater. The gamma group made up a significant percentage of total eubacteria at all samples accounting for 33, 42 and 23 % of eubacteria at Stns. 1, 3 and 6, respectively. It has been previously established that microbial communities in marine environments can be dominated by species from the gamma subclass of *Proteobacteria* [10, 13]. Glockner et al. [14] reported that the gamma subgroup appeared to be dominant in a freshwater bacterial community as well. Most of the water samples collected from the Satilla River contained high amounts of fine detrital material, and a large percentage of the bacteria were associated with detrital particles. Acinas et al. [1] found that the particle-associated bacterial communities in the

offshore Western Mediterranean Sea exhibited lower diversity than did the free-living bacteria, and was comprised almost exclusively of the gamma *Proteobacteria*. As measured by ISRT, the delta subgroup accounted for <8% of eubacterial cells in all samples. The sum of bacteria detected by the four group-specific primers was usually lower than the total number of cells detected by the eubacterial primer.

A second part to this investigation was to detect *in situ* gene expression of BTEX dioxygenases after natural bacterial communities had been exposed to toluene or xylene vapor for one week. About 20-30 % of DAPI stained cells expressed the BTEX degrading genes in Flask 1 (natural seawater that had been exposed to 0.2% toluene vapor) (Table 3). About 12-21% of the natural bacterial community responded to exposure of xylene vapor by expressing their BTEX dioxygenase genes (Flask 2 in Table 3). No positive BTEX signal was detected in the control samples. This experiment suggests that ISRT can be applied to detect *in situ* gene expression of natural microbial communities to help us understand microbial function corresponding to certain environmental changes.

Acknowledgements

We thank W. M. Sheldon for collecting Satilla water samples. We acknowledge support from the following funding agencies in The United States: Office of Naval Research, Marine Environmental Quality Program (N00041-93-0850); Department of Energy DE-FG02-97ER62451; National Science Foundation LMER (DEB-9412089) and OCE-9730602; and NOAA/Sea Grants Programs.

References

1. Acinas SG, Anton J, Rodriguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of Genes encoding 16S rRNA. App Environ Microbiol 65:514-522
2. Amann RI, Binder BJ, Olson RJ, Chisolm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial population. Appl Environ Microbiol 56:1919-1925
3. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143-169
4. Campbell L, Carpenter EJ, Iacono VL (1983) Identification and enumeration of marine chroococcoid cyanobacteria by immunofluorescence Appl Environ Microbiol 46:553-559
5. Chen F, Dustman WA, Moran MA, Hodson RE (1998) *In situ* PCR methodologies for visualization of microscale genetic and taxonomic diversities of prokaryotic communities. Ch. 3.3.9. In: Akkermans ADL, van Elsas JD, DeBruijn FJ (eds) Molecular Microbial Ecology Manual, suppl 3 Kluwer, Academic Publishers, The Netherlands, pp1-17
6. Chen F, Dustman WA, Hodson RE (In press) Detection of toluene dioxygenase gene and gene expression in *Pseudomonas putida* F1 in a toluene exposed seawater using *in situ* PCR and hybridization. Hydrobiologia

7. Chen F, Gonzalez JM, Dustman WA, Moran MA, Hodson RE (1997) *In situ* reverse transcription: an approach to characterize genetic diversity and activities of prokaryotes. Appl Environ Microbiol 63:4907-4913
8. Chen F, Hodson RE (In press) Viewing microbes with *in situ* molecular approaches, a MiniReview. In: Colwell R, Xu HS (eds) The Proceedings in Marine Biotechnology (ISPPMB'98), China Ocean Press
9. DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360-1362
10. DeLong EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol Oceanogr 38:924-934
11. Fuhrman JA, McCallum K, Davis AA (1992) Novel major archaebacterial group from marine plankton. Nature (London) 356:148-149
12. Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR (1988) Phylogenetic group specific oligonucleotide probes for identification of single microbial cells. J Bacteriol 170:720-726
13. Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) 345:60-63
14. Glöckner FO, Amann RI, Alfreider A, Pernthaler J, Psenner R, Trebesius K, Schleifer KH (1996) An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst Appl Microbiol 19:403-406
15. Gonzalez JM, Moran MA (1997) Numerical dominance of a group of marine bacteria in the α-subclass of the class *Proteobacteria* in coastal seawater. Appl Environ Microbiol 63:4237-4242
16. Hahn D, Amann RI, Zeyer J (1993) Detection of mRNA in *Streptomyces* cells by whole-cell hybridization with digoxigenin-labeled probes. Appl Environ Microbiol 59: 2753-2757
17. Hicks R, Amann RI, Stahl DA (1992) Dual staining of natural bacterioplankton with 4',6'-diamidino-2-phenelindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl Environ Microbiol 58:2158-2163
18. Hodson RE, Dustman WA, Garg RP, Moran MA (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl Environ Microbiol 61:4074-4082
19. Hönerlage W, Hahn D, Zeyer J (1995) Detection of mRNA of *nprM* in *Bacillus megaterium* ATCC 14581 grown in soil by whole-cell hybridization. Arch Microbiol 163:235-241
20. Lee S, Kemp PF (1994) Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. Limnol Oceanogr 39:869-879
21. Laniol BD, Giovanoni SJ (1997) Identification of bacterial cells by chromosomal painting. Appl Environ Microbiol 63:1118-1123
22. Porter J, Pickup R, Edwards C (1995) Flow cytometric detection of specific genes in genetically modified bacteria using *in situ* polymerase chain reaction. FEM Microbiology 134:51-56
23. Tecott LH, Barchas JD, Eberwine JH (1988) *In situ* transcription: specific synthesis of complementary DNA in fixed tissue sections. Science 240:1661-1664

24. Trebesius K, Amann RI, Lugwig W, Mühlegger K, Schleifer K-H (1994) Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl Environ Microbiol 60:3228-3235
25. Tolker-Nielson T, HolmstrØm K, Molin S (1997) Visualization of specific gene expression in individual *Salmonella typhimurium* Cells by *in situ* PCR. Appl Environ Microbiol 63:4196-4203
26. Ward BB (1982) Oceanic distribution of ammonium-oxidizing bacteria determined by immunofluorescent assay. J Mar Res 40:1155-1172
27. Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) 345:63-65
28. Zarda B, Amann RI, Schleifer K-H (1996) Identification of single bacterial cells using DIG-labeled oligonucleotides. In: Grünewald-Janho S, Keesey J, Leous M, Miltenburg RV, Schroeder C (eds) 2nd ed, Nonradioactive *in situ* hybridization application, Manual Boehringer Mannheim GmbH Biochemica, pp. 119-121