In situ reverse transcription-PCR and detection of *nirS* genes by Fluorescent *In Situ* Hybridization (FISH) to study denitrifying bacteria

GINA RODRÍGUEZ, CÉSAR E. CORDERO, GLADYS TORO, JACQUES A. VILLEFRANC, ERNIE PÉREZ AND ARTURO MASSOL-DEYÁ*

Department of Biology, University of Puerto Rico, P.O. Box 9012, Mayagüez, PR 00681

*Correspondence to Dr. Arturo A. Massol-Deyá, Email: amassol@uprm.edu

Abstract

Continental margin sediments constitute only about 10% of the total sediment surface area in the world's oceans, nevertheless they are the dominant sites of nitrogen (N) cycling. Recent studies suggest that the oceanic nitrogen budget is unbalanced, primarily due to a higher nitrogen removal rate in contrast to the fixation rate, and it has been suggested that denitrification activity contributes significantly to this imbalance. Although denitrification in marine environments has been studied intensively at the process level, little is known about the species abundance, composition, distribution, and functional differences of the denitrifying population. Understanding the diversity of microbial populations in marine environments, their responses to various environmental factors such as NO_3^-, and how this impact the rate of denitrification is critical to predict global N dynamics. Environmental Microbiology has the prompt to study the influence of each microbial population on a biogeochemical process within a given ecosystem. Culture-dependent and -independent techniques using nucleic acid probes can access the identity and activity of cultured and uncultured microorganisms. Nucleic acid probes can target distintict genes which set phylogenetic relationships, such as rDNA 16S, DNA gyrase (*gyrB*) and RNA polymerase sigma 70 factor (*rpoD*). In the other hand, the genetic capabilities and their expression could be tracked using probes that target several functional genes, such as *nirS, nirK*, *nosZ,* and *nifH*, which are genes involved in denitrification. Selective detection of cells actively expressing functional genes within a community using *In Situ* Reverse Transcription-PCR (ISRT-PCR) could become a powerful culture-independent technique in microbial ecology. Here we describe an approach to study the expression of *nirS* genes in denitrifying bacteria. Pure cultures of *Pseudomonas stutzeri* and *Paracoccus denitrificans*, as well as co-cultures with non-denitrifying populations were used to optimize the ISRT-PCR protocol. Cells grown on nitrate broth were harvested and fixed at both logarithmic (24-48 h) and stationary phase (7 days). Fixed and RNA protected$^{TM}_{cc}$ cells were spotted on microscope slides to optimize cell wall permeabilization conditions with lyzozyme and proteinase K. Subsequently, ISRT-PCR was performed with Nir*S* 1F and Nir*S* 6R primers using the QIAGEN® OneStep RT-PCR Kit. Amplification products within the cell were detected by Fluorescent *In Situ* Hybridization (FISH) at 40ºC overnight using a Cy3 labeled internal probe, specifically designed to detect the *nirS* gene. After hybridization, the cells were counterstained with DAPI and examined by confocal fluorescence microscopy. *P. stutzeri* cells treated with RNase and *Pseudomonas* G179 (a *nir*K denitrifying strain) were used as negative controls. Optimal cell permeabilization was achieved using 1 mg

ml^{-1} lyzozyme for 30 min and 2 µg ml^{-1} Proteinase K. RNase treated cells did not fluoresce after FISH, but were detectable by DAPI. Only nirS-type denitrifying cells in log phase (80-95% of total direct cell counts) were detected by this approach while fewer cells (5-10%) were detectable after 7 days in stationary phase. Co-cultures of *P. denitrificans* with a non-denitrifying isolate resulted in selective identification of target cells, thus supporting the potential use of this approach for gene expression analysis at the community level.

Introduction

Denitrification is a key process in nature that results in the transformation of oxidized forms of nitrogen to reduced dinitrogen gases including nitric oxide (NO) and nitrous oxide (N_2O). These gases contribute to ozone layer depletion and global warming (Ye et al., 1994). The ability for denitrification is widely distributed among a variety of taxa from major physiological groups including both, bacteria and archaea (Zumft, 1992). Although denitrification has been intensively studied at the process level, less is known as to its role in species abundance, composition, distribution, and functional dynamics. Investigating these processes at a species level is hindered to some extent by the dependence on culture-based techniques. Molecular techniques that assess genetic diversity at a community-level have been developed, given that culture-based techniques have limited our understanding of the diversity of naturally occurring prokaryotic communities (Amann et al., 1995). Molecular methods like denaturing gradient gel electrophoresis, clone libraries and terminal restriction fragment length polymorphisms based on 16S rDNA and rRNA are commonly used to determine the genetic diversity of a microbial community and to identify individual members within diverse communities. However, despite these advances, our understanding of the link between the functional activity of cells, environmental triggers and diversity is yet limited. Tools to evaluate active cells by non-culture techniques will improve our knowledge of microbial populations in natural environments as well as in artificial systems in order to better predict N dynamics.

Methods for monitoring gene expression in single cells have been developed to detect spatial-temporal gene activity in a complex community. These include gene fusions with reporter proteins that can be monitored microscopically. Although valuable, this technique involves genetically engineered organisms while functionally redundant populations could not be studied concurrently. Other alternative is to combine nucleic acid-based methods with *in situ* hybridization, as fluorescent *in situ* hybridization (FISH) allows single cell observation and phylogenetic identification simultaneously. An advantage of using such method for monitoring gene expression in single cells is that only partial sequence information is required. Nevertheless, this method requires the presence of hundreds of single stranded target sequences within an individual cell to produce a detectable signal. Usually, there are few target sites inside slow growing or stressed cells, therefore alternative methods have been introduced to increase the detection signal of single and low copy number genes. Enzymatic signal amplification (Pernthaler et al., 2002), multilabeled nucleotides contained by a DNA probe (Kenzaka et al. 2005), RNA-targeted primer extension (RPE) (Hodson et al., 1995, Kenzaka et al.,

2005) and *in situ* PCR (IS PCR) (Hodson et al., 1995, Chen et al., 1997) are complementary methods used to increase the sensitivity of probe-based detection assays.

Reverse transcription of mRNA to cDNA inside whole bacterial cells can be performed using a reverse transcriptase and a single complementary oligonucleotide primer. The *in situ* reverse transcription (ISRT) can be followed by PCR with primers targeted to specific functional activities (e.g. denitrifying genes). The *in situ* amplification and detection of specific target nucleic acid sequences are carried out inside individual cells rather than on bulk extracted nucleic acid, which are then visualized with an epifluorescent microscope (Nuovo, 1994). ISRT-PCR is a technology originally developed to detect DNA or RNA viruses inside eukaryotic cells (Bbagasra, 1990; Nuovo, 1994), but studies have shown that specific mRNA of functional genes such as *nahA* and *nifH* in prokaryotic cells can be detected as well (Hudson et al., 1995).

However, the ISRT-PCR approach is relatively new and still under development (Bagasra 1990, Haase et al., 1990, Chen et al., 1998). Nonspecific detection of incorporated labeled nucleotides during the amplification reaction is a major concern, resulting in false positive detection results (Hodson et al., 1995). To avoid non-specific PCR bias, an amplicon specific internal DNA probe could be designed and used for FISH. This downstream approach ensures a strong signal for only those amplified products that are complementary to the probe sequence. The integration of FISH with ISRT-PCR will allow more accurate profiling of *in situ* gene expression.

Despite the large diversity within denitrifying bacterial groups, a common element to these microorganisms is the nitrite reductase (Zumft 1997). Two structurally different nitrite reductases are found among denitrifiers, although never in the same cell (Zumft, 1997): One contains copper which is encoded by the *nirK* gene and the other contains a heme group encoded by the *nirS* gene (Braker et al., 1998). The continued transcription of the *nirS* operon requires the presence of nitrate or nitrite with a half-life of approximately 13 min (Hartig and Zumft, 1999). This work describes the successful specific detection of *nirS* gene expression in denitrifying populations using a pair of primers specifically targeted to the *nirS* gene and a monolabeled fluorescent probe internally complementary to the amplicons. Direct detection of active functional genes at the single-cell level constitutes an approach with significant implications to a better understanding of populations' distribution at the microscale level and necessary for modeling purposes. Although ISRT-PCR/FISH is laborious for routine analysis, this technique provides a highly sensitive and specific approach for detection of cells expressing specific functional genes within bacterial communities.

Materials and Methods

Bacterial samples and growth conditions. Denitrifying nirS-type cultures of *Pseudomonas stutzeri* (ATCC 14405), *P. aeruginosa* (DSM 6195), and *Paracoccus denitrificans* (ATCC 19367) were used in this study. The nirK-type strain *Pseudomonas* strain G-179 (M97294) was used as a negative control. All cultures were grown in nitrate broth (pH 7.6) with an inverted Durham tube at 27°C and constant shaking at 150 rpm.

Samples from mid exponential phase (24-48 h) and stationary phase (7 days) were collected and fixed immediately as described below.

Bacterial cell fixation. Fifty milliliters of each culture were used to harvest the cells by centrifugation at 5,000 rpm for 5 min. The cell pellet was resuspended in 500 µl of RNAprotect^{TM} Bacteria Reagent (QIAGEN, Valencia, CA) and incubated for 5 min at room temperature. After incubation, cells were gently resuspended in 50 ml of fresh filter sterilized 4% paraformaldehyde in a phosphate-buffered saline solution (PBS, 120mM NaCl and 2.7mM KCl in 10 mM [pH 7.6]) (Hodson et al., 1995) and incubated for 2 and 24 h at 4^{o}C. The cells were harvested and washed twice with 5 ml of 50% ethanol in 1X PBS (using centrifugation conditions described above) and then resuspended gently in 1 ml of absolute ethanol. Fixed cells were stored at -80°C until analysis but for a period no longer than one week. In order to (eliminate RNAse or to prevent mRNA degradation), all solutions used in fixation, permeabilization and *in situ* RT-PCR were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC, Sigma). Solutions were then incubated overnight at 37°C and autoclaved after the treatment to eliminate DEPC residues. All glassware was oven baked overnight at 240°C, and plasticware was thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA, and DEPC-treated dH_2O and autoclaved.

Prior to detection, cells were washed twice in 1X PBS and spotted (10 µl) onto RNase free *in situ* PCR glass slides (Applied Biosystems, Foster City, CA). Slides were incubated in 0.1% fresh DEPC for 12 min at room temperature, covered with 0.1% metaphor agarose for 10 min and washed with absolute ethanol (Pernthaler and Amman, 2004).

Cell permeabilization. A range of conditions were evaluated to optimize cell permeabilization by either electroporation or enzymatic treatment with lyzozyme/proteinase K. An overnight nuclease treatment was used to quickly assess the effects of these treatments on cell integrity and the effectiveness of the permeabilization step. One mg ml^{-1} RNase A and 100 U ml^{-1} DNase was used with a subsequent staining of samples with 50 µg µl^{-1} of 4',6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) for 5 min (Hodson et al., 1995). Removal of nucleic acids (lack of fluorescence) caused by the entrance of nucleases through the cell envelope was used as one criterion of permeabilization success. All samples were examined by epifluorescence microscopy (Olympus BH2-RFCA, Olympus, Japan).

Permeabilization by electroporation was evaluated as follows: a density of 10^7 fixed cells ml^{-1} were washed twice with double distilled water and subjected to successive pulses which electroporation time constants sum 10, 25, 50, 100 and 200 ms at 2.5 kV, 25 µF and 1000 Ω. Cold water and 1 mm gap electroporation cuvettes (Molecular Bioproducts) were used.

Enzymatic cell permeabilization effectiveness was evaluated by varying experimental conditions such as: time of exposure to paraformaldehyde (cell fixation) between 2 and 24 h, the time of exposure to 1 mg ml^{-1} lyzozyme from 15 to 30 min at 37°C and using proteinase K (0.5, 1, 2, 5 and 10 µg/ml final concentration) for 10 min at 37°C. Ten µl of

cell suspension were spotted on a glass slide and incubated with the enzymes which were removed by three consecutive washes with 1X PBS and a final wash with absolute ethanol. Protease was inactivated by heating the slides for 2 min at 94°C (Hodson et al., 1995). After complete processing of samples by ISRT-PCR and FISH detection (see below), the fluorescent intensity and cell morphology were used to identify the mildest but effective treatment for further analysis.

ISRT-PCR protocol. PCR amplification of *nirS* gene was performed using the QIAGEN OneStep RT-PCR Kit using a two-primer set, NirS 1F (5'-CCTAYTGGCCGCCRCART-3'; *P. stutzeri* position 763 to 780) and NirS 6R (5'-CGTTGAACTTRCCGGT-3': *P. stutzeri* position 1638 to 1653)(Braker et al., 1988). The spotted cells were then covered with 50 µl of reaction mixture (30 µl of RNase free water, 10 µl of 5X RT-PCR Buffer, 2 µl of dNTP Mix 10 mM each, 0.6 µM of each primer, and 2 µl of RT-PCR Enzyme Mix) and sealed using the assembly tool (Perkin Elmer, Norwalk, CT). The reactions were performed in an automated *in situ* PCR thermal cycler (Gene Amp In Situ PCR System 1000 Perkin Elmer, Norwalk, CT). The following temperatures and time profiles were used: reverse transcription at 50°C for 30 min, a hotstart activation step at 95°C for 15 min, initial denaturation at 94°C for 5 min, followed by 10 cycles of melting at 94°C for 30 sec, annealing at 56°C for 40 sec and extension at 72°C for 40 sec. This sequence was followed by 25 cycles using similar conditions but 54°C as the annealing temperature. A final extension was performed at 72°C for 7 min. After amplification, the slides were washed twice with 1X PBS.

Detection of amplified gene products by fluorescent *in situ* hybridization (FISH). After ISRT-PCR, slides were incubated in denaturation buffer (0.5 M NaOH/1.5 M NaCl) at 25°C for 10 min. Subsequently, samples were transfered to neutralizing buffer (1 M Tris-HCl (pH 8.0) 1.5 M NaCl) for 5 min at 25 °C and then serially dehydrated with ethanol (50, 80 and 100%). The slides were prehybridized in DIG Easy Hybridization Solution (Roche Applied Science, Germany) for one hour. The cyanine dye (Cy3) labeled PSTUT probe (5'-TTCCTBCAYGACGGCGG-3'; *P. stutzeri* position 1251 to 1267) was boiled for 5 min and kept at 4°C. The probe was diluted 1:10 in DIG Easy hyb solution for a final concentration of 75 ng μl^{-1}. The spotted cells were then covered with 30 µl of the hybridization mixture and incubated overnight at 40°C in the *in situ* PCR thermal cycler. After hybridization, the slides were washed twice with pre-warmed hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 7.2] and 0.01% SDS) at 48°C for 20 min. Finally, slides were washed with washing buffer (900 mM NaCl/100 mM Tris-HCl [pH 7.2] for 5 min at 25°C. Samples were counterstained with DAPI as described previously and examined under epifluorescence microscopy (Olympus BH2-RFCA, Olympus, Japan). Photographs were taken using a digital SPOT Insight Color Camera and the corresponding SPOT Advanced software V 3.2 (Diagnostic Instruments, USA). UG-1 filter (Olympus, Japan) and XF108-2 filter set (Omega Optical, Brattleboro, Vermont) were used to visualize DAPI and Cy3 signals, respectively. Samples were examined by Laser Confocal Microscopy model Olympus FluoView 300.

Results and Discussion

Cell Permeabilization. Enzyme permeabilization and electroporation were evaluated for their effectiveness to make stable pores in the cell wall large enough to allow access of both RNase (approx. 13,000 Da) and DNase (approx. 33,000 Da) inside the cells while the overall morphology remains intact. Both enzymatic treatments and electroproporation were performed after cells were fixed with 4 % paraformaldehyde. This pre-treatment rigidly fix cell wall proteins preventing closure of pores after permeabilization (Nuovo, 1996). The ability of RNases and DNases to cross the cell envelop after electroporation for 100 ms is shown in figure 1. Treatment with 50 ms was insufficient to promote effective pore sizes for entry of enzymes in all cells while 200 ms destroyed the cell. DAPI staining proved that these enzymes destroyed nucleic acids inside treated cells after an overnight incubation. Ineffective permeabilization can result in false negatives, since PCR and FISH reactants cannot entry the cell. On the contrary, excessive permeabilization can result in either false positives, resulting from leaking of amplicons into "negative" cells, or false negatives, from leaking of amplicons out of "positive" cells. Our results showed that electroporation, a faster and more economical procedure, could be employed for ISPCR. A limitation of electroporation, is the low starting cell density (10^7 cells ml^{-1}) requirement from which 10 µl are used to spot *in situ* PCR slides, further reducing the available cell density. Since cells are expected to be lost during washes and further sample processing, cell concentration by centrifugation or other method could be necessary. This additional step can potentially compromise the integrity of already weaken cells in the ISRT PCR protocol.

Currently, enzyme treatment is the permeabilization method most widely used for *in situ* applications. This protocol consists of a lyzozyme and proteinase K treatment. The most effective lyzozyme treatment (1 mg ml^{-1}) was achieved after 30 min of incubation. In turn, proteinase K at 0.5 µg ml^{-1} was effective with cells fixed for 2 hours in 4% paraformaldehyde while 2 µg ml^{-1} were required with 24 hours fixed cells. Nouvo (1996) documented needs for higher concentrations of pronase when digesting cells fixed for longer periods. Nonetheless, the finest permeabilization condition was achieved with 24 hour fixed cells and incubated for 30 min with a lyzozyme solution and 2 µg ml^{-1} of proteinase K. These permeabilization conditions were chosen for the ISRT-PCR protocol.

ISRT-PCR. Despite controlled fixation and permeabilization, direct IS-PCR or ISRT-PCR using labeled nucleotides in the reaction will yield non-specific amplification products and therefore a high frequency of false positive results (Massol, unpublished data; Komminoth and Long 1993 and 1995; Chen et al., 1999). Weak primer annealing during PCR amplification can also result in a number of non-specific amplicons with detectable signal. Other causes for false positives in direct IS-PCR/RT-PCR could be attributed to: (i) incorporation of labeled nucleotides into cellular DNA by the repair mechanism of DNA polymerase, (ii) endogenous priming, in which endogenous DNA or RNA fragments act as primers for PCR amplification, (iii) unstable binding between fluorochrome and dUTP during heating and cooling cycles of PCR which releases fluorochrome that might act like a general stain for all cells, and finally (iv) binding of fluorescently-labeled dUTP to the cellular components inside cells due to high

temperatures during PCR cycles (Komminoth and Long 1995; Chen et al., 1999). Therefore, the detection of *in situ* PCR products could be more effectively assessed by performing a FISH step using a fluorescently labeled DNA oligonucleotide probe targeting an internal region of the amplicon.

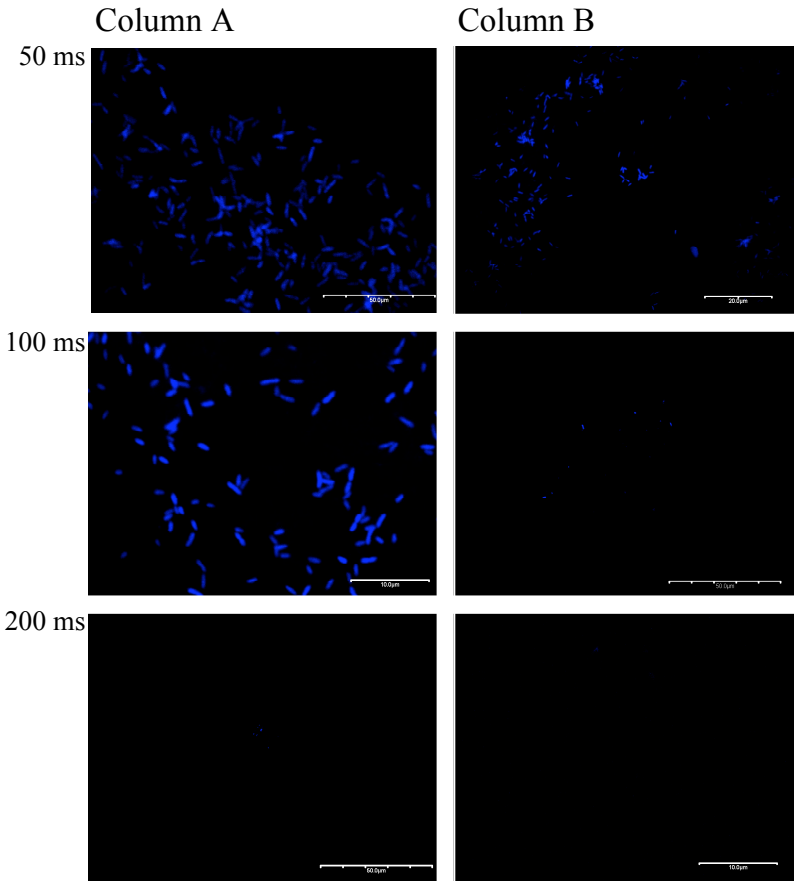

FIG. 1. DAPI stained cells of *Pseudomonas stutzeri* observed by confocal microscopy after electroporation for 50, 100 and 200 ms. Column A: Cell integrity after electroporation: Column B: Electroporated cells treated overnight with RNase and DNase.

Prior to *in situ* detection, *nirS* gene amplification using the NirS 1F and NirS 6R primers was tested with pure DNA (PCR) and RNA (RT-PCR) samples. A PCR product of 890 bp was obtained only for bacterial strains harboring the *nirS* gene (Braker et al., 1998). After testing the specificity of the primers with pure cultures, ISRT-PCR amplification was performed and successfully achieved for exponentially growing denitrifying cultures of *P. denitrificans, P. aeruginosa* and *P. stutzeri* while background signals were observed in all negative controls including *Pseudomonas* spp. G-179 and *P. stutzeri* treated with RNase before ISRT-PCR (Figure 2).

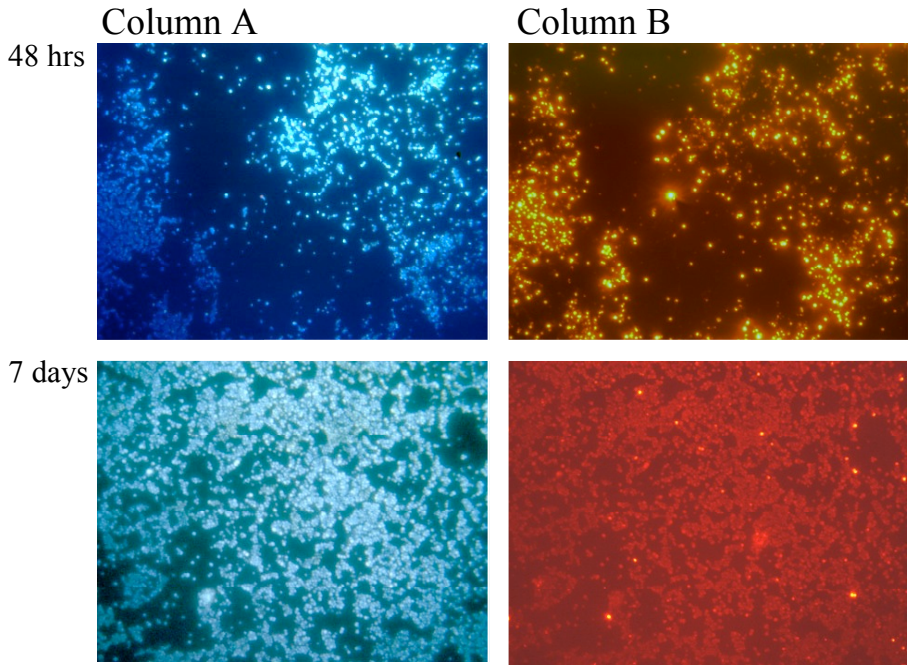

FIG. 2. Cells of *P. denitrificans* observed by epifluorescence microscopy. Column A: DAPI counterstained cells. Column B: ISRT-PCR FISH (Cy3-nirS [orange] labeled cells).

With exponentially growing pure cultures (as determined by observable gas production and positive NO_3 reduction test after 48 h of incubation), a large number of cells (80-95%) were detected by ISRT-PCR when compared to the total cell counts observed by DAPI staining. However, duplicates of stationary phase cultures (7 days of incubation) resulted in a dramatic decrease in the number of cells detectable by this approach (5-10%). The observed reduction of detectable cells between 48 h cultures and 1-week starving cells was physiologically expected since expression of *nirS* is maintained at a low oxygen tension if nitrate or nitrite is present with an available electron donor (Härtig and Zumft 1999). A reduction in the number of cells at stationary phase detected by ISRT-PCR and the lack of detection in active denitrifying cultures treated with RNAse prior to analysis are both evidence that mRNA, not chromosomal DNA, is being the primary template (target) for reverse transcription and subsequent amplification reaction. Therefore, this demonstrates that *nirS* gene ISRT-PCR targets and detects actively produced mRNA by induced cells that codifies for a key enzyme of the denitrification process.

When ISRT-PCR was tested with a co-culture of *P. stutzeri* and *Escherichia coli* (non-denitrifying rod) in stationary phase, only *Pseudomonas* cells were detected by fluorescence microscopy (Figure 3). The frequency of pseudomonas-like cells detected in the co-culture was similar to cell counts observed with pure cultures in stationary phase.

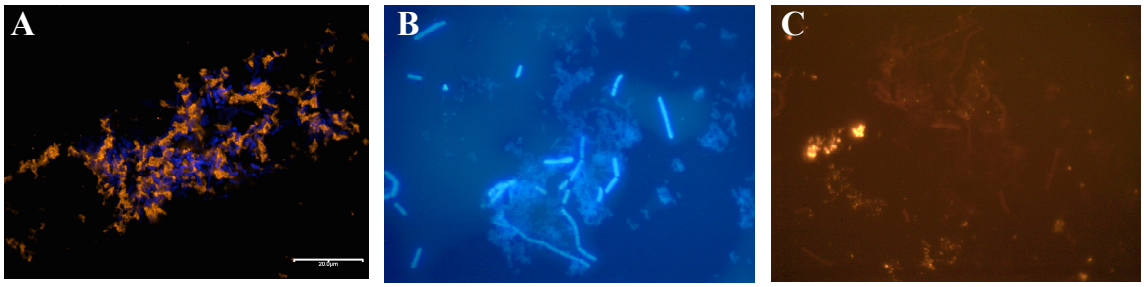

FIG. 3. Confocal images of ISRT-PCR FISH of *nirS* in a co-culture of *P. stutzeri* with a non-denitrifying rod. (A) Cells in logarithmic phase (Cy3-nirS orange) was superposed on the corresponding DAPI signal (blue), (B) DAPI stained cells in stationary phase, and (C) Cy3-nirS.

Our results show that ISRT-PCR with denitrifying cultures could not account for all possible cells detected by direct staining in the sample. Although it is probable that not all cells in exponentially growing cultures are actually active, false negatives could also result for various reasons as previously observed by Long and co-workers (1993). Possible explanations for the false negative results include: (1) cell to cell variations in the amplification efficiency due to differences in membrane permeability, (2) associated proteins causing non-accessibility of mRNA, (3) cell fixation or permeabilization biases in older cultures and (4) loss of amplification products during washing steps in the detection procedures.

Although target bacterial cells could be successfully detected using this technique, its functionality cannot be fully described until an environmental sample processing approach is developed to maintain the integrity of local microbial assemblages. Processing in the laboratory such as centrifugation or filtration alters the natural distribution of microbes in the sample. Nutrient rich environments such as bioreactors, agricultural or bioremediation environments could be more suitable for ISRT-PCR studies while an improved technique with special needs for cell fixation and permeabilization might be necessary for oligotrophic sites (environments). Regardless of the current and possible limitations, analysis of mRNA as an indicator of gene expression by ISRT-PCR is a tool that can enhance our understanding of active functional groups in the environment. Furthermore, ISRT-PCR provides a direct indication of specific gene expression at the time of sampling and therefore can be linked to specific physicochemical conditions in the ecosystems or study sites. Evaluation of expression of putative new genes discovered by gene libraries could be tested by this approach thus complementing traditional DNA/RNA exclusive techniques. In cases where active cells might be limited by resources, chemical amendments can be used to evaluate triggers of gene expression and the potential density of cells that actively respond to the environmental perturbation.

Acknowledgments

We thank James M. Tiedje and the Center for Microbial Ecology (CME) at Michigan State University for their support to this research. We are also grateful to Ricardo Maggi, Gesche Braker and Veronica Gruntzing for providing technical support and José Almodóvar for his contribution with confocal laser analysis at the UPRM Center for Microscopy. This research was funded by the US Department of Energy (USDOE) Biotechnology Investigations Ocean Margin Program (BI-OMP) grant DE-FG02-04ER63738.

References

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995 . Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143-169.

Bagasra O. 1990. Polymerase chain reaction *in situ*. Amplification. (March): 20-21.

Braker, G., A. Fesefeldt, and K.-P. Witzel. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64: 3769-3775.

Braker, G., J. Z. Zhou, L. Y. Wu, A. H. Devol, and J. M. Tiedje. 2000. Nitrite reductase genes (*nirS* and *nirK*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 66: 2096-2104.

Chen F., Dustman W. A., and Hodson R. E. 1999. Microscopic detection of the toluene dioxygenase gene and its expression inside bacterial cells in seawater using prokaryotic *in situ* PCR. Hydrobiologia. 401: 131-138.

Chen F., Gonzalez J. M., Dustman W. A., Moran M. A., Hodson R. E. 1997. In Situ Reverse Transcription, an Approach To Characterize Genetic Diversity and Activities of Prokaryotes. Appl. Environ. Microbiol. 63: 4907-4913.

Chen F., W. A. Dustman, M. A. Moran, and R. E. Hodson. 1998. *In situ* PCR methodologies for visualization of microscale genetic and taxonomic diversities of prokaryotic communities. Molecular Microbial Ecology Manual. A. D. L. Akkermans, J. D. Van Elsas, and F. J. De Brujin (ed). Kluwer Academic Publishers, Norwell, MA. 3.3.9: 1-17.

Devol A. H. 1991. Direct measurement of nitrogen gas fluxes from continental shelf sediments. Nature. 349: 319-321.

Haase A. T., E. F. Retzel, and K. A. Staskus. 1990. Amplification and detection of lentiviral DNA inside cells. Proc. Natl. Acad. Sci. U.S.A. 37: 4971-4975.

Härtig E. and Zumft W.G. 1999. Kinetics of *nirS* Expression (Cytochrome *cd*1 Nitrite Reductase) in *Pseudomonas stutzeri* during the Transition from Aerobic Respiration to Denitrification: Evidence for a Denitrification-Specific Nitrate- and Nitrite-Responsive Regulatory System. J. of Bacteriol. 181: 161-166.

Hodson, R. E., W. A. Dustman, R. P. Garg, and M. A. Moran. 1995. *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl. Environ. Microbiol. 61: 4074-4082.

Hodson, R. E., W. A. Dustman, R. P. Garg, and M. A. Moran. 1995. In situ PCR for visualization of microscale distribution of specific genes and gene product situ and

dynamics of bacteria on limnetic organic aggregates (lake snow). Appl. Environ. Microbiol. 62:1998–2005.

Kenzaka T., Tamaki S., Yamaguchi N., Tani K., Nasu M. 2005. Recognition of Individual Genes in Diverse Microorganisms by Cycling Primed In Situ Amplification. Appl. Environ. Microbiol. 71: 7236-7244.

Komminoth, P., and A. A. Long. 1993. In situ polymerase chain reaction: an overview of methods, applications and limitations of a new molecular technique. Virchows Arch. B Cell Pathol. 64: 67-73.

Komminoth, P., V. Adams, A. A. Long, J. Roth, P. Saremaslani, R. Flury, M. Schmid, and P. U. Heitz. 1994. Evaluation of methods for hepatitis C virus (HCV) detection in liver biopsies: comparison of histology, immunohistochemistry, *in situ* hybridization, reverse transcriptase (RT) PCR and *in situ* RT PCR. Path. Res. Pract. 190: 1017-1025.

Long, A. A., P. Komminoth, and H. Wolfe. 1993. Comparison of indirect and direct *insitu* polymerase chain reaction in cell preparations and tissue sections. Histochemistry 99: 151-162.

Middleburg, J. J., K. Soetaer, P. M. J. Herman, and C. H. R. Heip. 1996. Denitrification in marine sediments: A model study. Global Biogeochem. Cycles. 10: 661-671.

Nogales, B., K. N. Timmis, D. B. Nedwell, and A. M. Osborn. 2002. Detection and Diversity of Expressed Denitrification Genes in Estuarine Sediments after Reverse Transcription-PCR Amplification from mRNA. Appl. Environ. Microbiol. 68: 5017-5025.

Nuovo, G. J. 1994. PCR *in situ* hybridization: protocols and applications. 2nd ed. Raven Press, New York.

Pernthaler A. and Amann R. 2004. Simultaneous Fluorescence In Situ Hybridization of mRNA and rRNA in Environmental Bacteria. Appl. Environ. Microbiol. 70: 5426-5433.

Pernthaler A., Pernthaler J., and Amann R. 2002. Fluorescence In Situ Hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria. Appl. Environ. Microbiol. 68: 3094-3101.

Rösh C., Mergel A., and Bothe H. 2002. Biodiversity of Denitrifying and Dinitrogen-Fixing Bacteria in an Acid Forest Soil. Appl. Environ. Microbiol. 68: 3818-3829.

Tiedje, J. M. 1988 . Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. *In*In A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York, N.Y.

Walsh J. J. 1991. Importance of the continental margins in the marine biogeochemical cycling of carbon and nitrogen. Nature 350:53-55.

Ward, B. B. 1996. Nitrification and denitrification: probing the nitrogen cycle in aquatic environments. Microb. Ecol. 32: 247-261.

Ward B. B., and A. R. Cockcroft, and K. A. Kilpatrick. 1993. Antibody and DNA probes for detection of nitrite reductase in seawater. J. Gen. Microbiol. 139: 2285-2293.

Yamamoto S., Kasai H., Arnold D. L., Jackson R. W., Vivian A., and Harayama S. 2000. Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from thenucleotide sequences of *gyrB* and *rpoD* genes. Microbiology 146: 2385–2394.

Ye, R. W., B. A. Averill., and J. M. Tiedje. 1994. Denitrification: Production and consumption of nitric oxide. Appl. Environ. Microbiol. 60: 1053- 1058.

Zumft, W. G. 1992. The denitrifying prokaryotes, p. 554-582. *In* A. Balows (ed.), The prokaryotes: a handbook of the biology of bacteria: ecophysiology, isolation, identification, applications. Springer-Verlag, New York, N.Y.

Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61:533-616.