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Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR

X.-Y. Qiu[a,b], R.A. Hurt[a], L.-Y. Wu[a,b], C.-H. Chen[c], J.M. Tiedje[b], J.-Z. Zhou[a,b,*]

[a]*Environmental Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6038, United States*
[b]*Center for Microbial Ecology, Michigan State University, East Lansing, MI, 48824, United States*
[c]*Life Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6378, USA*

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Abstract

We developed a quantitative competitive PCR (QC-PCR) system to detect and quantify copper-denitrifying bacteria in environmental samples. The primers were specific to copper-dependent nitrite reductase gene (*nirK*). We were able to detect about 200 copeis of *nirK* in the presence of abundant non-specific target DNA and about 1.2×10^3 *Pseudomonas* sp. G-179 cells from one gram of sterilized soil by PCR amplification. A 312-bp *nirK* internal standard (IS) was constructed, which showed very similar amplification efficiency with the target *nirK* fragment (349 bp) over 4 orders of magnitude (10^3–10^6). The accuracy of this system was evaluated by quantifying various known amount of *nirK* DNA. The linear regressions were obtained with a R^2 of 0.9867 for 10^3 copies of *nirK*, 0.9917 for 10^4 copies of *nirK*, 0.9899 for 10^5 copies of *nirK* and 0.9846 for 10^6 copies of *nirK*. A high correlation between measured *nirK* and calculated *nirK* (slope of 1.0398, R^2=0.9992) demonstrated that an accurate measurement could be achieved with this system. Using this method, we quantified *nirK* in several A-horizon and stream sediment samples from eastern Tennessee. In general, the abundance of *nirK* was in the range of 10^8–10^9 copies g soil^{-1} dry weight. The *nirK* content in the soil samples appeared correlated with NH_4(N) content in the soil. The activities of copper-denitrifying bacteria were evaluated by quantifying cDNA of *nirK*. In most of sample examined, the content of *nirK* cDNA was less than 10^5 copies g soil^{-1} dry weight. Higher *nirK* cDNA content (>10^6 copies g soil^{-1} dry weight) was detected from both sediment samples at Rattlebox Creek and the Walker Branch West Ridge. Although the stream sediment samples at the Walker Branch West Ridge contained less half of the *nirK* gene content as compared to A-horizon sample, the activities of copper-denitrifying bacteria were almost 600 times higher than in the A-horizon sample.

Keywords: Copper-denitrifying bacteria; Quantitative competitive PCR; Denitrification

1. Introduction

Denitrification is a respiratory process of bacteria in which oxidized nitrogen compounds are reduced to

* Corresponding author. Tel.: +1-423-576-7544; fax: +1-423-576-8646.
E-mail address: zhouj@ornl.gov (J.-Z. Zhou).

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nitrogen gases (NO, N_2O and N_2). The great interest of this process is its important role in practical applications: denitrification causes nitrogen losses in agricultural soils (Firestone, 1982) and marine environments (Seitzinger, 1990), and accumulation of greenhouse gases (NO and N_2O) in the atmosphere. Also, denitrification is used in waste treatments (Tiedje, 1988) and bioremediation of environmental pollutants (Fries et al., 1994).

Accurate detection and quantification of denitrifying bacteria from natural habitats will enable us to better understand environmental importance of denitrification. However, it has been a challenge since dinitrifiers are widely distributed to diverse phylogenic groups. PCR amplification of nitrite reductase genes (*nirS* and *nirK*) has been applied to detect and identify denitrifiers from culture collection and a variety of environmental samples (Braker et al., 1998; Gruntzig et al., 2001; Hallin and Lindgren, 1999; Michotey et al., 2000). *nirS* encodes nitrite reductase containing heme *c* and *d1*; *nirK* encodes nitrite reductase containing copper. The two types of Nir are functionally and physiologically equivalent (Zumft, 1997). Generally, *nirS* is more widely distributed while *nirK* is found in only about 30% of denitrifiers and is present in a wider range of physiological groups (Coyne et al., 1989). Recently, PCR-based quantitation on cd_1-dentrifying bacteria from environmental samples has been reported (Gruntzig et al., 2001; Michotey et al., 2000). Quantitative measurement of copper-denitrifying bacterial from natural habitats will enable us to monitor entire denitrifying population, thus can better understand the dynamics of the denitrification process in a functional community.

Quantitative competitive PCR (QC-PCR) is one of the most widely used approaches for quantitation of nucleic acids. The assay is based on competitive co-amplification of a specific target sequence together with known amounts of an internal standard in one reaction tube. The internal standard (IS) has the same primer recognition sites with the specific template, thus is co-amplified with the specific target template. Quantitation of the specific target template is obtained by comparing the intensities of PCR amplification products from the specific template with those from the IS. Since any variations during a PCR reaction will have the same impact on the amplification of both target template and IS, the final ratio between amplified target template and IS should reflect their initial ratio. Ever since the technique was first described (Beckerandre and Hahlbrock, 1989; Gilliland et al., 1990; Wang et al., 1989), it has been widely used for quantitation of cellular DNA and RNA as well as viral and bacterial nucleic acids (Fox et al., 1992; Kaneko et al., 1992; Li and Drake, 2001; Piatak et al., 1993; Ramakrishnan et al., 1994; Rezzonico et al., 2003; Stieger et al., 1991; Zhang et al., 2002). Competitive PCR has also been successfully used to quantify specific groups of bacteria from environmental samples (HallierSoulier et al., 1996; Johnsen et al., 1999; Kondo et al., 2004; Leser et al., 1995; Mendum et al., 1999; Mesarch et al., 2000; Rudi et al., 1998; Stephen et al., 1999; Watanabe et al., 1998). It has been demonstrated that it is a simple and robust way for measuring unculturable microorganisms.

In this study, we report the development of quantitative competitive PCR system for quantitation of copper-denitrifying bacteria from environmental samples.

2. Materials and methods

2.1. Organisms and environmental samples

Bacterial strains and isolates were grown overnight in nutrient broth (Difco, Detroit, MI) at 30 °C. For quantification cDNA of *nirK*, *Pseudomonas* sp. strain G179 was inoculated in nutrient broth supplemented with 0.05% potassium nitrate and cultured anaerobically. *Escherichia coli* transformants were grown in LB broth amended with kanamycin (50 μg/ml).

Soil and stream sediment samples collected at the Great Smoky Mountains and Oak Ridge National Laboratory Environmental Research in Eastern Tennessee were homogenized by manual mixing, frozen in liquid nitrogen, transported on dry ice, and stored at −40 °C. For soil characterization, samples were air-dried and weighed. Nitrogen content was measured using a PE2400 Series II CHNS/O Analyzer (Elmer, Norwalk, CT).

2.2. Nucleic acids extraction and purification

Genomic DNA of pure culture or isolate was isolated with a sodium dodecyl sulfate (SDS)-based

method and quantified using a spectrophotometer (Zhou et al., 1996). Genomic DNA and total RNA of soil and stream sediment sample were extracted, purified, and quantified using the simultaneous DNA and RNA extraction method (Hurt et al., 2001). Plasmid DNA was prepared using a Wizard mini-Preps DNA Purification System (Promega, Madison, WI) and measured spectrophotometrically and verified on agarose gel. The diluted plasmid DNA was measured by Picogreen dye (Molecule Probes, Eugene, OR) using a Microtiter® Plate Fluorometer (The Microtiter®).

2.3. Primer design

Primers specific to the *nirK* were designed by aligning available *nirK* genes downloaded from GenBank (Table 1) using CLUSTALW. Seventy out of twenty two nucleotides in the forward primer *nirK* F (5′ TCATGGTCCTGCCGCGYGACGG3′) was conserved. Y was a mixture of C and T. In the reverse primer *nirK* R (5′GAA CTT GCC GGT NGC CCA GAC), 18 out of 21 nucleotides were conserved. N was the mixture of nucleotides A, T, C and G.

2.4. PCR amplification

PCR amplification condition was first optimized for primer and $MgCl_2$ concentrations. All PCR amplifications were accomplished using the optimal conditions with "hot start" (Daquila et al., 1991) in a 20-μl volume containing 1× *Taq*DNA polymerase buffer [10 mM Tris–Cl, 50 mM KCl (pH 8.3)], 1.2 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 6 pmol of each primer, 100 μg/ml BSA, and 0.5 U of *Taq* DNA polymerase (Promega). PCR reaction was performed in an automated thermal cycler (GeneAmp 9700, PE Applied Biosystem, Branchburg, NJ) with an initial denaturation at 95 °C for 2 min, followed by various cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min.

2.5. Specificity and sensitivity test

Genomic DNA of several denitrifying strains, marine isolates and *E. coli* (Table 2) were isolated as described above. Ten nanograms of gDNA was used for PCR amplification with 35 cycles. The PCR products were resolved on 1.8 % of agarose gel and detected by ethidium bromide staining. All reactions were performed in triplicates.

The sensitivity of the *nirK* primers was first evaluated using a 10-fold serial dilution of *Pseudomonas* sp. strain G-179 genomic DNA (from 10 ng to 10 fg) in the presence of 10 ng of non-specific target DNA (*Shewanella oneidensis* MR-1 genomic DNA). Since the genome of *Pseudomonas* sp. strain G-179 was unknown, we used a 10-fold serial dilution of cloned *nirK* (from 2×10^7 to 200 copies) in the presence of 10 ng of non-specific target DNA (*S. oneidensis* MR-1 genomic DNA) to evaluate the detection limit. To test the sensitivity on detecting copper denitrifier, *Pseudomonas* sp. strain G-179 and *E. coli DH5a* in late exponential growth phase were enumerated by acridine orange counts. One milliliter each of 10-fold serial dilutions of G-179 suspension plus 1.0 ml of *E. coli* cells (8×10^8cells per ml) was added to 0.5 g of the sterile soil, and incubated at room temperature for 1 h

Table 1
Strains used for designing *nirK*-specific primers

Strains	GenBank accession number	JT 562 (5′ to 3′)	JT 563 (5′ to 3′)
A. cycloclastes	Z48635	TCATGGTGCTGCCGCGCGACGG	GAACTTGCCGGTCGCCCAGAC
A. faecalis strain S-6	398121	– – – – – – – – – – – – – – – – G – –	– – – – – – – – – – – – – – – – – – –
Pseudomonas sp. G179	AF083948	– – – – – – C – – – – – – – – – – T	– – – – – – – – – – A – – – – – –
P. aureofaciens	Z21945	– G – – – – – – – – – – – – C – – T –	– – – – – – – – – – G – T – – – –
Rhodobacter sphaeroides	U62291	– – – – – – – – – – – – – – — – – –	– – – – – – – – – – TT – – – – –
NirK primers		TCATGGTGCTGCCGCGYGACGG	GAACTTGCCGGTNGCCCAGAC

Table 2
Bacterial strains used for specificity testing

Strains or isolates	Source	Subgroups	Nir types	PCR amplification with *nirK*-specific primers
Pseudomonas sp. Strain G-179	M97294	Alpha	copper	+
Bacillus azotoformans	ATCC 29788	Gram-positive	copper	+
VT116	R.Ye	Alpha	copper	+
Corynebacterium nephridii	ATCC 11425	Gram-positive	copper	+
Pseudomonas aureofaciens	ATCC13985	Gamma	copper	+
Alcaligenes faecalis	ATCC8750	Beta	copper	+
C3-2 (marine isolate)[a]	L.Wu	Alpha	copper	+
B4-6 (marine isolate)[a]	L.Wu	Gamma	copper	+
D3-16 (marine isolate)[a]	L.Wu	Gram positive	copper	+[b]
A3-5 (marine isolate)[a]	L.Wu	Gamma	cd_1	0
B2-2 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
B9-12 (marine isolate)[a]	L.Wu	Gamma	cd_1	+[c]
C10-5 (marine isolate)[a]	L.Wu	Gamma	cd_1	+[c]
D4-14 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
D7-6 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
D8-12 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
D9-1 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
E4-2 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
F8-5 (marine isolate)[a]	L.Wu	Gamma	cd_1	−
F9-1 (marine isolate)[a]	L.Wu	Gamma	cd_1	0
Shewanella oneidensis MR-1	ATCC700550	Gamma	none	−
Escherichia coli K12	DSM 498	Gamma	none	−

+: visible band of expected size; 0: weak band of any other size; −: no visible band.
[a] Isolated from Washington Margin (Braker et al., 1998).
[b] Band is visually weak.
[c] Band is visually very weak.

prior to DNA extraction. The sterile soil was produced by autoclaving the soil three times at 121 °C for 1 h (Zhou et al., 1997). All treatments were performed in triplicates.

2.6. Construction of nirK internal standard (IS)

The IS of *nirK* was generated using an approach similar to that of Siebert and Larrick's (1993). Briefly, low stringency conditions (annealing at 45 °C) were used in the amplification with composite primers (*nirK* CF: 5′TCATGGTCCTGCCGC GTGACGGcacccagaaggctgagggc3′; *nirK* CR: 5′GAACTTGCCGGTAGCCCA GACcaccgggattgaccacggt3′). These specific primers (cacccagaaggctgagggc and caccgggattgaccacggt) were targeted to nucleotides 201–219 and 451-469 of *nirK* gene of *Pseudomonas* sp. strain G-179. Amplified IS (312 bp) were cloned to a pCR™ vector using a TA Cloning® kit (Invitrogen, CA), and confirmed by PCR with *nirK*-specific primers. The PCR product of IS is 37 bp shorter than *nirK* fragment.

The amplification efficiency of IS was evaluated using a cloned *nirK* from *Pseudomonas* sp. strain G-179 in the presence of 10 ng of genomic DNA of *Shewanella oneidenase* MR-1. Equal molar quantities of *nirK* and IS (from 10^3 to 10^6 copies of each) were mixed and subjected to PCR amplifications. PCR products were examined every 2 cycles after 20 cycles. The total cycle number varied with the template concentrations. A total of 30 cycles was used for 2×10^5 and 2×10^6 copies of templates, 34 cycles with 2×10^4 copies and 36 cycles with 2×10^3 copies of templates. Amplified *nirK* and IS were resolved on a 2 % TBE agarose gel. The intensity of each band was analyzed using EagleSight software (STRATAGENE, La Jolla, CA). To calibrate the integrated intensity due to the size difference between *nirK* and IS, a standard containing equal amount of *nirK* and IS (quantified by Picogreen dye) was run in parallel with the assaying samples. The correction factor based on the ratio of IS to *nirK* from standards was used to normalize the intensity of the each band.

2.7. Competitive PCR

Quantitation of *nirK* by competitive PCR was carried out as follows: First, 10-fold serial dilutions of IS co-amplified with the sample DNA (10–100 ng of genomic DNA). The dilution of IS which gave roughly equal amplification with the target sample was used as a reference point for making two-fold serial dilutions of IS. Sample DNA was then co-amplified with the two-fold serial dilutions of IS. The initial ratios of IS to *nirK* in the sample DNA were calculated based on the calibrated intensity (same as above) of amplified IS and *nirK* fragments on the agarose gel. The $\log_2$ of each ratio was plotted as a function of the $\log_2$ of the added IS, which yields a linear plot. The interpolation on the plot for a *Y* value of 0 gives the number of *nirK* in the sample DNA. PCR amplification was same as described with an annealing temperature between 58–65 °C.

2.8. Reverse transcription PCR (RT-PCR)

Reverse transcription was performed in a 20-μl of reaction volume, containing 1–2 μg of total RNA, 200 U of Superscript II RNase H^- reverse transcriptase (Life Technologies, Grand Island, NY), and 20 pmol of primer *nirK*R at 42 °C for 55 min according to manufacture's instructions. After inactivated at 72 °C for 15 min, the samples were treated with RNase H (Life Technologies) and purified using a QIAGEN DNA purification kit. Transcribed cDNA was quantified by competitive PCR as described above.

3. Results

3.1. Specificity

The specificity of the primers was examined with denitrifiers from a diverse phylogenetic group (Table 2). The 349-bp PCR amplicons were observed for all copper denitrifiers including three α-proteobacteria, one β-proteobacterium, two γ-proteobacteria and three Gram-positive bacteria. No specific amplifications were observed in *P. stutzeri* isolates from marine environments, which contain *nirS* genes (Table 2). A very weak *nirK* amplification was observed in the marine isolates B9-12, which is closely related to *Halomonas variabilis* SW 32, and C10-5, which is closely related to *Marinobacteria* sp. Both isolates gave very strong amplification with *nirS* specific primers.

3.2. Sensitivity tests

Detection limit was first evaluated using a dilution series of *Pseudomonas* sp. strain G179 DNA. On agarose gel, the detection level was 1 pg (Fig. 1A). Assuming G-179 only contains one copy of *nirK*, and 5 fg per genome, we could detect 200 copies of *nirK* with this pair of primers. The consistent result was obtained by spiking a dilution series of cloned *nirK* (4249-bp plasmid DNA) into 10 ng of *S. oneidensis* MR-1 DNA. Specific amplification was observed from 1 fg of plasmid DNA, which was equivalent to 200 copies of *nirK* (Fig. 1B). Specific amplification was also observed in the soil seeded with 5.9×10^2 of G179 cells (Fig. 1C), thus, we can detect about 1.2×10^3 G-179 cells from one gram of soil.

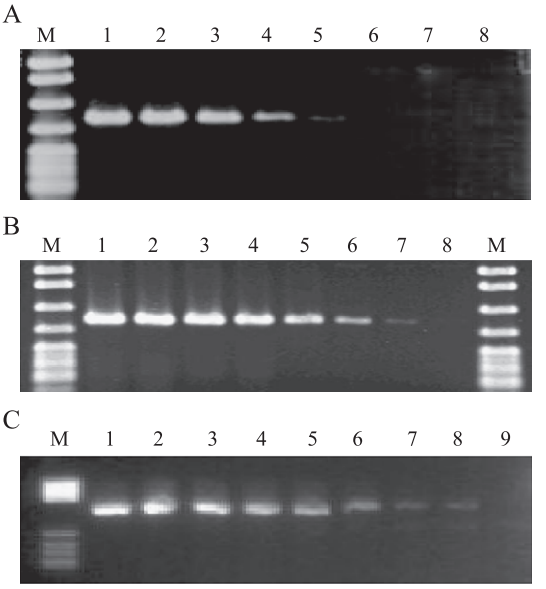

Fig. 1. Sensitivity test of *nirK* primers with genomic DNA of *Pseudomonas* sp. strain G-179 (A); plasmid borne *nirK* in the presence of 10 ng of *S. oneidensis* MR-1 DNA (B); genomic DNA extracted from soils seeded with various amount of *Pseudomonas* sp. strain G-179 cells along with 10^8 *E. coli* cells (C). A: M: DNA Marker; lanes 1–8: 10, 1 ng, 100, 10, 1 pg, 100, 10 fg, negative control (no DNA); B: M: DNA Marker; lanes 1–8: 1 ng, 100, 10, 1 pg, 100, 10, 1 fg, and negative control (no DNA); C: M: DNA Marker; lanes 1–9: 7.5×10^4, 3.8×10^4, 1.9×10^4, 9.4×10^3, 4.7×10^3, 2.3×10^3, 1.2×10^3, 5.9×10^2, and negative control (soil contains only *E. coli* cells).

3.3. Internal standard (IS) of nirK

To avoid the formation of heteroduplex between IS and *nirK* fragment, we used the upstream region of the target *nirK* fragment to construct the IS. Since IS has different intervening sequence and size from the target *nirK* fragment, the amplification efficiency was examined by co-amplifying equal molar quantities of IS and the target *nirK* fragment. Similar amplification efficiencies were obtained with the template over 4 orders of magnitude examined (Fig. 2). The PCR reactions reached plateau periods after 26, 30, 32 and 34 cycle, respectively, when 10^6, 10^5, 10^4 and 10^3 copies of each IS and *nirK* were used as templates. To obtain accurate

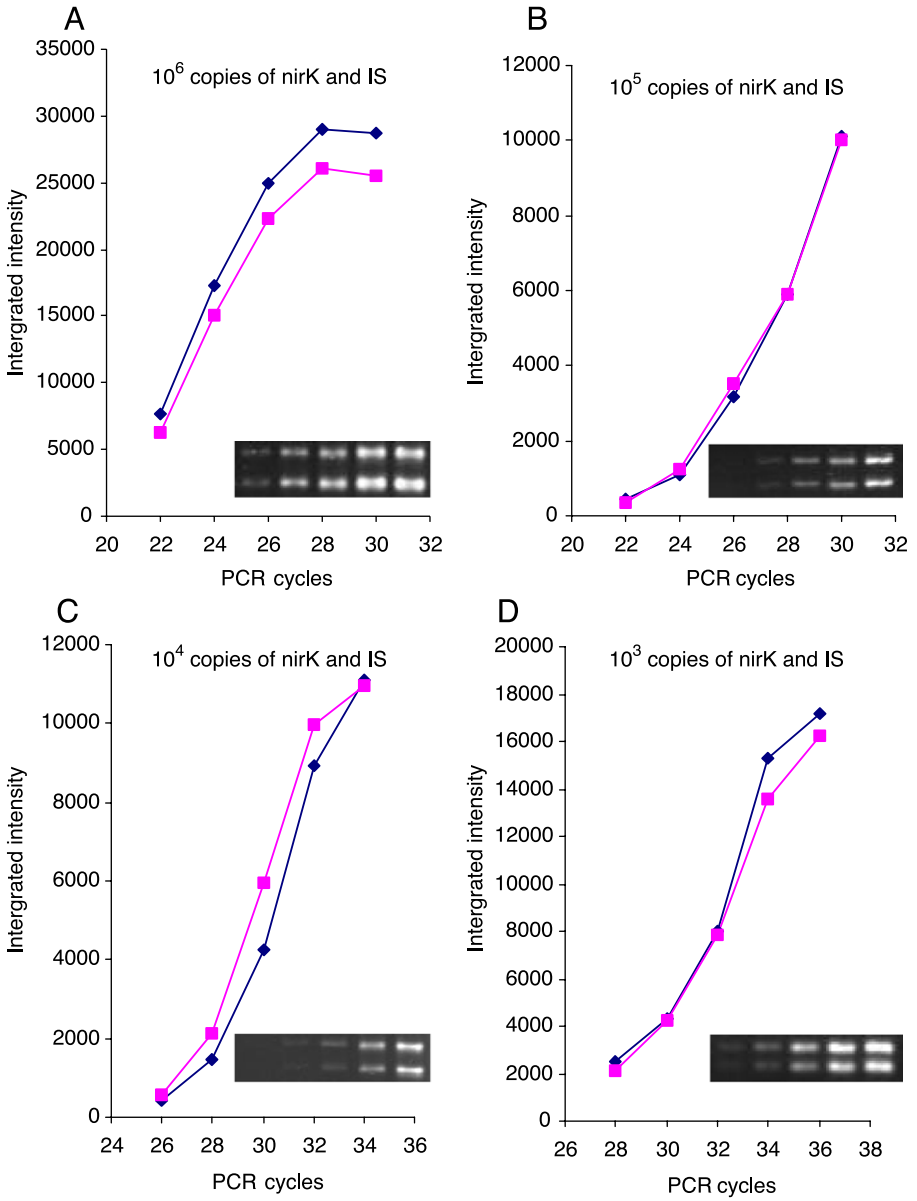

Fig. 2. Co-amplification of *nirK* and IS. (A) 10^6 copies of *nirK*and IS; (B)10^5 copies of *nirK*and IS; (C): 10^4 copies of *nirK* and IS; (D) 10^3 copies of *nirK*and IS. Similar plot patterns were observed in replicate experiments.

quantitation, competitive PCR should be performed within exponential phase (Wiesner et al., 1993).Thus, competitive PCR for quantitation of 10^6, 10^5, 10^4 and 10^3copies of *nirK* should be performed for less than 26, 30, 32 and 34 cycles, respectively.

3.4. Accuracy and quantitation limit

The accuracy of competitive PCR was evaluated by using known amount of cloned *nirK* fragments, ranging from 10^3 to 10^6 copies. To evaluate how

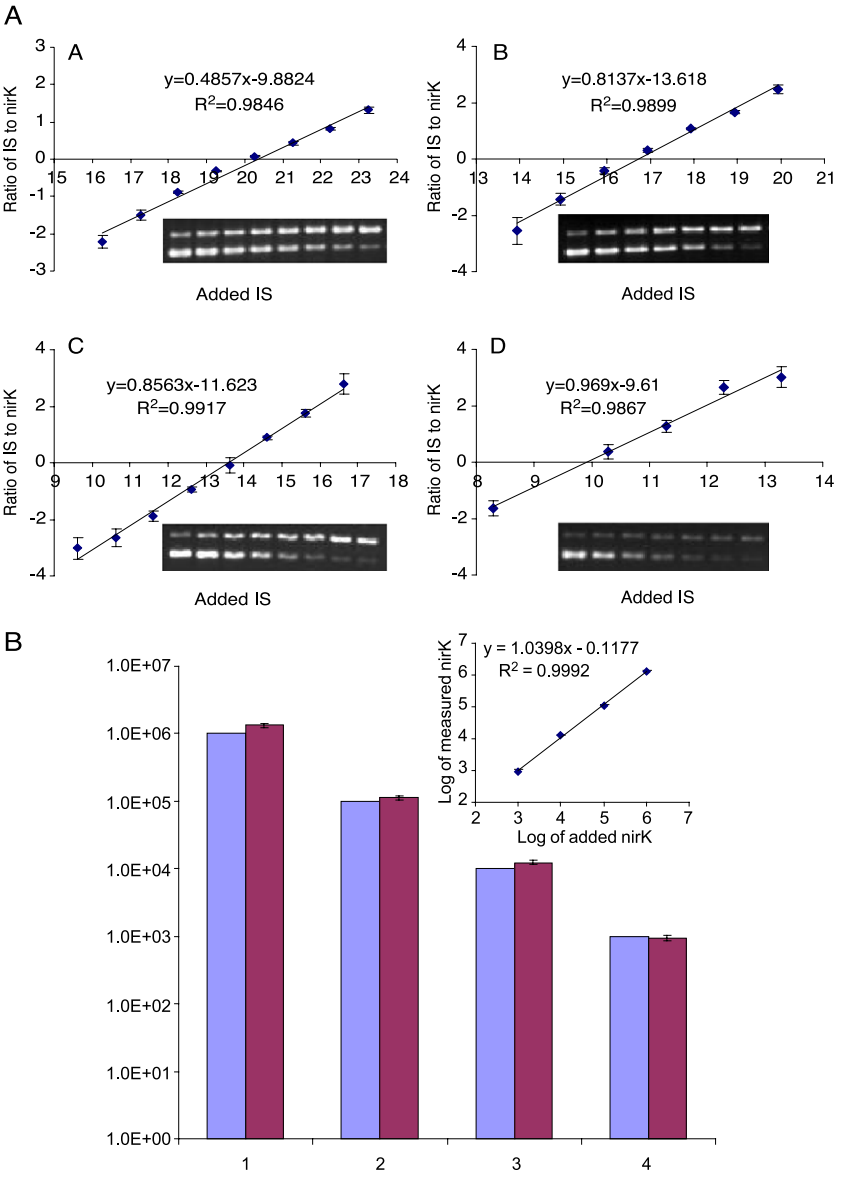

Fig. 3. Accuracy of competitive PCR. A: quantitation of 10^6 (A), 10^5 (B), 10^4 (C) and 10^3 (D) copies of plasmid borne *nirK* by competitive PCR in presence of 10 ng of gDNA from *S. oneidensis* MR-1. The agarose gel profiles showed two-fold serial dilution of IS co-amplified with 10^6 copies of *nirK* for 25 cycles (A), 10^5 copies of *nirK* for 27 cycles (B), 10^4 copies of *nirK* for 31 cycles (C) and 10^3 copies of *nirK* for 33 cycles (D); error bar stands for standard deviations (n=3). (B) Correlation between measured *nirK* (1,331,550±81,020; 111,957±8584; 12,455±798; 947±104, n=3) and calculated *nirK*(10^6, 10^5, 10^4, and 10^3).

heterogeneous templates will affect the accuracy of quantification, all reactions included 10 ng of genomic DNA from a non-denitrifying bacterium, *S. oneidensis* MR-1. To maintain the amplification within exponential phase, a total of 25, 27, 31 and 33 cycles was used for quantitation of 10^6, 10^5, 10^4 and 10^3 copies of *nirK* fragment, respectively. A two-fold dilution series of IS were used to co-amplify with constant amount of *nirK*. Linear regression of ratios of IS to *nirK* ($\log_2$) and added IS ($\log_2$) was obtained for all assays with a R^2 of 0.9867 for 10^3 copies of *nirK*, 0.9917 for 10^4 copies of *nirK*, 0.9899 for 10^5 copies of *nirK* and 0.9846 for 10^6 copies of *nirK* (Fig. 3A). The correlation between measured and added values was extremely high (slope=1.0398), which indicated a precise quantitation over a wide range (Fig. 3B). When there was less than 10^3 copies of *nirK* fragment in the competitive PCR reaction, no good linear regression was achieved (R^2<0.9), thus, we were unable to quantify less than 10^3 *nirK* using this system.

3.5. Detection and quantification of nirK from environmental samples

We used this system to monitor *nirK* genes from a variety of soils and sediment samples. *NirK*-specific amplifications were observed in many samples. The abundance of copper-denitrifying bacteria in several A-horizon and stream sediment samples was estimated using competitive PCR (Table 3). Among six A-horizon samples examined, the highest content of the *nir*K gene was observed in a sample recovered from the Walker Branch West Ridge (5.7×10^9 copies g soil^{-1} dry weight), which contained the highest mineral nitrogen content (Table 3). The total DNA, RNA and *nirK* gene contents of these soils are significantly correlated with NH_4–N content (R^2=0.85, P=0.01; R^2=0.71, P=0.05; R^2=0.83, P=0.05), but not with NO_3-(N) content. The *nirK* content in Walker Branch sediment samples was relatively higher than other sediment samples (Table 3). No significant relationships were observed between total DNA, RNA or *nirK* gene content and NH_4-(N) or NO_3-(N) content in the sediment samples.

The relative activities of copper-denitrifying bacteria were estimated by quantifying the cDNA of *nir*K. cDNA of *nirK* in soil samples were generally lower than 10^5 copies per gram of dry soil. The highest cDNA content was observed in the stream sediment samples obtained from Rattlebox Creek and Walker Branch West Ridge (about 10^6 copies of cDNA g soil^{-1} dry weight). Although stream sediment samples at the Walker Branch West Ridge contained less half of the *nir*K gene content as compared to A-horizon sample, the expressed *nirK* gene was almost 600 times higher than in the A-horizon sample. No significant

Table 3
Quantification of copper-denitrifying bacteria in environmental samples

Collection site[a]	Sample type	N-content (μg/g or ml)		DNA yield[b] (μg/g soil)	RNA yield[b] (μg/g soil)	*nirK* (copies/g soil)	*nirK* cDNA (copies/g soil)
		NH_4-(N)	NO_3-(N)				
NC	A Horizon	0.98	0.55	97.4	13.3	1.6×10^9	<6.6×10^{4c}
MB	A Horizon	0.57	0.01	52.5	16.7	7.9×10^8	<8.4×10^{4c}
MC	A Horizon	1.25	0.01	42.9	15.2	6.6×10^8	<7.6×10^{4c}
RC	A Horizon	0.91	0.00	107.4	20.1	1.5×10^9	<1.0×10^{5c}
WBE	A Horizon	1.19	0.00	45.8	<1	6.0×10^8	<5×10^{3c}
WBW	A Horizon	2.78	0.00	435.1	56.1	5.7×10^9	4×10^3
NC	Stream sediment	<0.01	0.60	23.0	3.0	3.5×10^8	<1.5×10^{4c}
MB	Stream sediment	<0.01	0.20	49.4	3.9	8.4×10^8	2×10^4
MC	Stream sediment	<0.01	0.07	60.6	<1	9.7×10^8	<5×10^{3c}
RC	Stream sediment	<0.01	0.02	40.7	1.4	6.1×10^8	1×10^6
WBE	Stream sediment	<0.01	0.05	96.6	11.9	1.7×10^9	<6×10^{4c}
WBW	Stream sediment	<0.01	0.04	137.1	22.5	2.3×10^9	2.5×10^6

[a] NC: Noland Creek; MB: Minni Ball; MC: Mossy Creek; RC: Rattlebox Creek; WBE: Walker Branch East Ridge; WBW: Walker Branch West Ridge.
[b] Yield after the final purification.
[c] Could not obtain further close estimations due to PCR inhibitions or not enough template RNA.

correlations can be drawn between the cDNA content of *nirK* and the chemical characteristics of samples such as total carbon and nitrogen content.

4. Discussion

Primer sets for detection of nitrite reductase gene in the denitrifying bacteria have been reported (Braker et al., 1998; Hallin and Lindgren, 1999). The combination of those primers (*nirK*and *nirS*) allowed qualitatively detection of the denitrifying bacteria in a variety of environmental samples (Braker et al., 1998; Braker et al., 2000). However, to quantify by PCR technique, the capacity of the primers has to be tested extensively in order to obtain reliable interpretable results. Specificity and sensitivity are the two major factors that affect the PCR based detection and quantification, thus have to be examined carefully.

NirK-specific primers used in this study were in one of the conserved regions reported by Braker et al. (1998). Thirteen bases of forward the primer (n*irK*F) were identical to the 3′ end of primer *nirK*2F, whereas reverse primer (*nirK*R) was identical as *nirK*3R (Braker et al., 1998). To achieve high specificity, mismatches at the 3′ ends of the primers were designed to be minimal for *nirK* sequences. For detecting broad range of *nirK* genes, the promiscuous nucleotide analogues Y (for T, C) and N (for A, G, C, T) were used at the positions exhibiting sequence variations among the selected genes. The degenerated primers are particular useful since the denitrification is widely spread over the phylogenic groups. We used a relative high annealing temperature (60–65 °C) to minimize the non-specific amplifications in the quantitation reactions.

Strong *nirK* amplification observed in eight of copper-denitrifying strains examined, including α, β, γ-proteobacteria and Gram-positive bacteria. Since the primers are very similar to *nirK*2F and *nirK*3R developed by Braker et al. (1998), we expect successful amplifications from strains tested by the same authors. *NirK*primers also showed good matches with the putative *nirK* gene from *Ensifer* sp. 4FB6 (GB# AY078248), *Ochrobactrum* sp. 4FB13 (GB# AY078252) and *Pseudomonas mendocina* (GB# AY078255). Among 10 marine isolates that were characterized as cd_1-denitrifying bacteria, non-specific amplification was observed in isolates A3-5 and F9-1. Although a very weak band as the same size as *nirK* was observed from the isolates B9-12 and C10-5, it disappeared when the annealing temperature increased to 65 °C. Specific detection was also obtained by DNA hybridization using the *nirK* probe generated from strain *Pseudomonas* sp. strain G-179 (data not shown). All these results indicated that the primers used in this study are specific to *nirK* gene, and are suitable to detect copper-denitrifying bacteria from natural samples.

The sensitivity of PCR detection is dependent on the annealing efficiency of primers to the templates, amplification efficiency, and the detection methods. With the primers, 1–100 pg of template DNA from pure culture can be reliably detected, which is equivalent to about 2×10^2–2×10^4 cells, assuming 5 fg of DNA per cell. The sensitivity was not affected in the presence of 10 ng of non-specific DNA (about 2×10^6 molecules). This detection limit is slightly higher than the primers set used for quantitation of cytochrome cd_1-dependent denitrifying bacteria (Michotey et al., 2000). The detection sensitivity appears to be dependent on the sequence conservation between the primers and the templates. For instance, the *nirK* genes from marine isolates C3-2 are about 66.7% identical to that of *Pseudomonas* sp. strain G179. The detection sensitivity with the primer set decreased about 100-fold compared to strain G179. The sensitivity on environmental samples may vary much due to the variations on diversity of *nirK*and abundance of copper denitrifiers in a natural habitat. Furthermore, humic substances co-extracted with nucleic acids from soil or sediment samples may inhibit PCR reaction, which may affect the detection limit. To achieve the best sensitivity, the PCR amplifications were performed under optimal conditions with lower primer concentrations to avoid the primer dimmer formation (Zhou et al., 1997).

The greatest advantages of competitive PCR are simple, precise, and lower cost. A critical step in a competitive PCR assay is the construction of a good internal standard. The more similar the amplification efficiencies of competitor and target DNA are, the more accurate quantitation can be achieved. Although a variety of methods have been used for construction of internal standard (Zimmermann and Mannhalter, 1996), many intrinsic problems limited its application. Those problems can be summarized as follows: (1): the

amplification efficiency of competitor was assumed the same as target gene since the two molecules were very similar, which is not always true (Diviacco et al., 1992); (2): additional step such as the restriction digestion was required for resolution of internal standard from the target DNAs (Beckerandre and Hahlbrock, 1989; Gilliland et al., 1990), which lowers the accuracy of the assay; (3): quantitative accuracy is affected by the formation of heterduplexes between the target DNA and competitors (Beckerandre and Hahlbrock, 1989; Piatak et al., 1993). Here we adapted the approach described by Siebert and Larrick (1993) for construction the *nirK* internal standard. This method is simple and has a great range (any region of the gene other than the targeted sequence) to select internal standard. Since the internal standard has different intervening sequence from the target DNA, the size and GC contents of the IS as well as the sequence similarity between target DNA and IS are the essential factors affecting the co-amplification efficiencies. To obtain the best IS, the size and GC content should be as similar as possible to the targeted DNA fragment, whereas sequence similarity between IS and target DNA should be as low as possible (avoid the formation of heteroduplex). For simplification of the method, size difference between target and IS should allow easy separation on the agarose gel. The co-amplification efficiency has to be determined experimentally. The IS of *nirK* we designed in this study showed very similar amplification efficiency to the *nirK* target sequence over 4 orders of magnitudes in the presence of 10 ng of non-target DNA (about 2×10^6 molecules). The co-amplification efficiencies did not change when the proportion of target molecules to non-target molecules varies from 0.3 to 0.001 (Fig. 2). Reliable quantitation can be achieved when there are more than 10^3copies of *nirK* in the sample.

Theoretically, competitive PCR is not cycle dependent (Gilliland et al., 1990). However, this is true only when amplification efficiencies of target and competitor are identical. Our results clearly indicate that performing the assay within the exponential phase is important for precise quantitation. Ability to discriminate a two-fold difference in the final PCR products decreased when PCR reached post-exponential or stationary phase.

Competitive reverse transcription-polymerase chain reaction (RT-PCR) has been used to measure mRNA levels (Beckerandre and Hahlbrock, 1989; Gilliland et al., 1990; Kaneko et al., 1992; Piatak et al., 1993; Siebert and Larrick, 1992; Stieger et al., 1991). Direct quantitation of message RNA requires the constructing of an RNA internal standard with the same reverse transcription efficiency to the sample RNA (Zimmermann and Mannhalter, 1996). The sample RNA will be mixed with a series dilution of internal standard RNA. The sample RNA and IS will be co-reverse-transcripted and co-amplified. The amount of mRNA in the sample will be obtained by comparing the amplified target molecules and internal standard. Thus, this method requires relative large amount of target RNA for a single quantitation. Quantitation of cDNA is an alternative way to estimate the level of mRNA. It has been used to quantify nitric oxide synthase mRNA in mouse cells by Siebert and Larrick (1992). This method is simple and require less amount of sample RNA, which is especially practical to estimate the genes or bacteria activities from environmental samples. Since reverse transcription efficiency of sample RNA may varied among different samples, the comparison of gene or bacteria activity using cDNA may be more meaningful when applying to compare the same sample with different treatments or samples with similar chemical characteristics. To compare the gene activities between different samples, the effect of RT efficiency has to be considered.

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